STRUCTURAL STUDIES ON LAMINARIN AND RELATED POLYSACCHARIDES

- by -

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ABSTRACT OF THESIS

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Title of Thesis Structural Studies on Laminarin and Related Polysaccharides.

This thesis deals with structural studies on the polysaccharides laminarin and lichenin, both of which are polymers of β-D-glucose.

Periodate oxidation of model compounds has been studied and the results generally confirm and extend those of previous workers. A method has been devised for determination of the degree of polymerisation of laminarin by oxidation of the derived laminaritol.

Several samples of laminarin have been examined, including two which were extracted under non-acidic conditions to reduce the possibility of inadvertent degradation.

The presence of mannose as a constituent of laminarin, which was reported by two groups of workers, has not been confirmed. Only minute quantities (ca. 0.2%) of mannose have been detected in hydrolysates of laminarin samples, and it is considered that this sugar, in common with fucose, arises from contaminating carbohydrate and is not structurally significant.

1-Substitution, as opposed to 1,2-disubstitution postulated by other workers, of the mannitol residues in laminarin has been established by selective periodate oxidation and by the isolation of ethylene glycol from laminarin polyalcohol.

Mild acid hydrolysis of laminarin polyalcohol, to cleave acetal but not glucosidic linkages, has furnished no evidence for the presence of 1,6- inter-residue, as opposed to inter-chain, linkages in laminarin. Oligosaccharide fragments were absent from such hydrolysates. The absence of 1,6- inter-residue linkages from a sample of methylated laminarin was confirmed by the absence of 2,3,4-trimethyl glucose, as a constituent sugar.

The main features of several laminarin samples have been examined by periodate oxidation, and the most prominent variation

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observed was in the degree of branching. An "insoluble" sample possessed 0.3 branch points per molecule in contrast to 1.6 for a "soluble" sample. The former figure has been confirmed by estimation of the glycerol content of the derived polyalcohol. It appears, therefore, that spontaneous precipitation of laminarin from aqueous solution is associated with a low degree of branching.

The heterogeneity of an "insoluble" sample has been examined by gel-filtration, spontaneous precipitation and dialysis but marked fractionation was not obtained.

The absence of erythritol glycosides of laminaridextrins from partial acid hydrolysates of lichenin polyalcohol has shown that the 1,3- linkages present in lichenin occur entirely in isolation rather than as sequences. Such hydrolysates, however, contained one unidentified component. Mild acid hydrolysis at room temperature produced several fragments in which acetal linkages persisted, indicating that their stability was greater than that previously indicated by other workers.

Fractionation of polyhydric alcohols on strongly basic ion-exchange resin in the hydroxide form has been observed. Elution occurred in order of increasing molecular size, the reverse of the order found by others in similar experiments using carbohydrates on ion-exchange resins in salt form.

ACKNOWLEDGEMENTS

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CONTENTS Page.

Contents (con.) Page.

FIGURES

Figures (con.) Facing page.

INTRODUCTION

Laminarin was first described by Schmiedeberg[1] in 1885. The name is currently applied (e.g. Quillet,[2] 1958) to the glucans which may be readily extracted by hot water from various species of Phaeophyceae. Structural studies, however, have been carried out only on laminarins extracted from Laminaria and Eisenia species and these will be reviewed here.

In 1909 Krefting and Torup[3] reported the extraction of laminarin from L. digitata. The product gave no coloration with iodine, was non-reducing, was laevorotatory and was hydrolysed by hydrochloric acid to glucose. Hydrolysis could also be carried out by an enzyme present in the plant but not by "ptyalin, amylopsin or diastase". The laminarin was freely soluble in water at 40-50°, but soluble only to the extent of 0.05% at 20° and virtually insoluble at 0°.

Kylin[4] isolated laminarin fractions with different solubilities in water and ethanol and measured their optical rotation and degree of polymerisation (D.P.).

$[\alpha]_D$ in water	Degree of polymerisation
-32.5°	6
-22.5°	12
-13.5°	17

Laminarin is therefore polydisperse.

Both glucose and galactose, which were identified as their osazones, were found in the hydrolysate of an extract from L. flexicaulis (= digitata[5]) by Mme. Gruzewska[6]. She also observed[7] that laminarin could be dialysed through a collodion membrane and that it retrograded from solution, for which latter process oxygen appeared to be necessary.

Colin and Ricard[8,9] prepared laminarin from L. flexicaulis. Repeated washing and precipitation with ethanol was required to remove free mannitol. The solubility was given as 21g. per 100g. of water and the D.P. as 6 to 7. Only glucose was detected after hydrolysis, which indicated that the galactose which was previously reported[6] must have arisen from a contaminating oligo- or polysaccharide.

An extensive investigation was carried out by Barry[10] in 1938 on laminarin extracted from L. digitata, and purified by spontaneous deposition from aqueous solution. Barry attempted to show fractionation of laminarin by measuring the optical rotation of successive deposits from an aqueous solution. In contrast to Kylin's fractionation by precipitation with ethanol, fractionation could not be demonstrated. Variation of specific rotation with concentration was

demonstrated however, a steady increase in laevorotatory power from -6.81° to -14.64° being observed as the concentration was decreased from 5.44% to 0.24%. Reduction of hypoiodite by laminarin indicated one reducing glucose group per ca. 36 glucose residues. The reducing power was independent of the concentration of the laminarin solution, and therefore the observed change in rotation with concentration was ascribed to physical aggregation which did not affect the reducing glucose residues. That glucose was the only constituent sugar was confirmed by polarimetric measurements on a hydrolysate and the fact that the optical activity of a hydrolysate could be completely removed by fermentation with yeast. The suggestion was made that laminarin might be sulphated to a small extent.

Acetylation[11] of laminarin from L.hyperborea (formerly known as L.cloustoni; see e.g. Parke[12]) gave a triacetate from which laminarin still possessing the ability to precipitate spontaneously from aqueous solution could be recovered by saponification. Methylation and subsequent methanolysis and hydrolysis afforded 2,4,6-tri-o-methyl D-glucopyranose in good yield indicating the presence of 1,3-linkages, and the low rotations of laminarin and its derivatives, which resembled those of cellulose and lichenin rather than

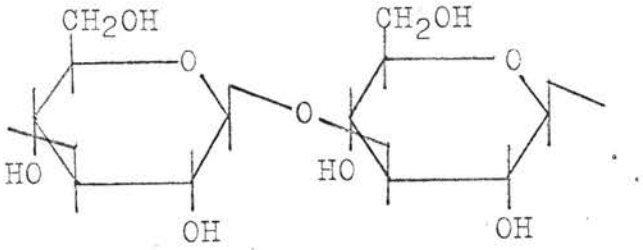

Fig. 1. THE MAIN LINKAGE IN LAMINARIN.

those of starch and glycogen, indicated that these linkages had a β configuration (Fig.1).

The isolation of laminaribiose was reported by Barry[13] in 1941. The osazone and the free sugar were both isolated after partial enzymic and acid hydrolyses. The osazone was shown to be the same as that isolated by Zechmeister and Toth[14] who had previously studied yeast glucan. Emulsin was shown to hydrolyse the disaccharide to glucose.

The reduction of periodic acid by laminarin was found[15] to be very much less than that by cellulose or starch, confirming the 1,3- mode of linkage; the observed reduction being ascribed to oxidation of end-groups. On the assumption that glycol cleavage occurred only at the non-reducing terminal end units, a method for their assay was devised[16]. The dialdehyde produced by periodic acid oxidation was further oxidised by bromine to a dicarboxylic acid, which was estimated by titration with sodium hydroxide solution and by ignition of its silver salt. It was appreciated that a definite proportion of carboxyl groups was introduced at the reducing glucose end unit by the bromine oxidation, and a correction for this was applied. A chain length of 16 was obtained by this method. Estimation of tetramethyl glucose from methylated laminarin was also attempted,

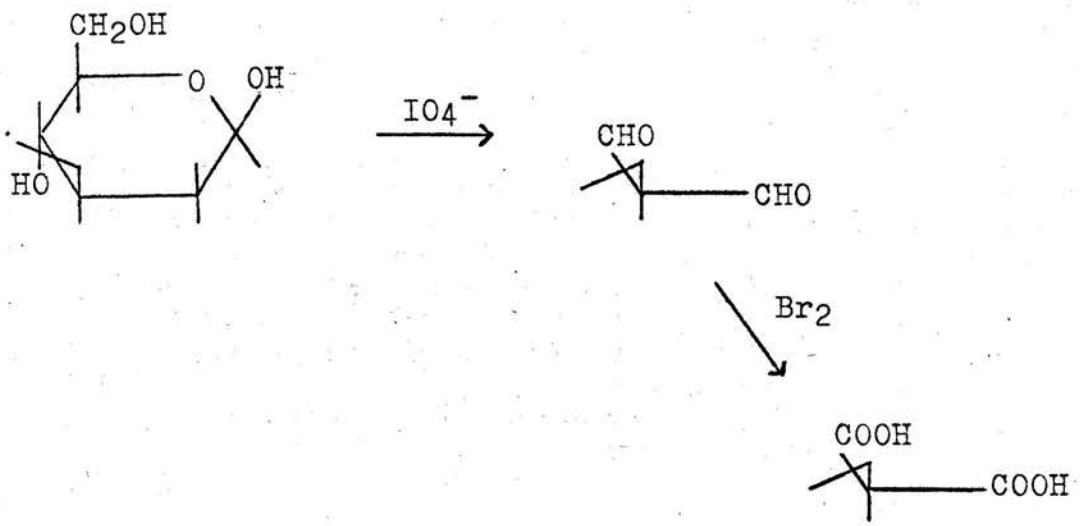

Fig. 2. OXIDATION OF THE REDUCING GLUCOSE TERMINAL RESIDUE.

giving a chain length of 73 but the discrepancy was ascribed to the experimental inadequacies of the procedure for separation and estimation of the methylated sugars.

Barry[17] applied his degradation method to laminarin but did not report any check to the step-wise removal of the non-reducing end group as appeared to occur in the case of yeast glucan.

Structural studies were then continued by Connell, Hirst and Percival[18] who criticised Barry's chain length determination by suggesting that oxidation by periodic acid would convert a free reducing group into a malondialdehyde derivative. Oxidation of this structure by bromine would introduce two carboxyl groups (Fig.2), whereas Barry had envisaged the introduction of only one carboxyl group. It was therefore suggested that a chain length of 20 might be more accurate. This result is further open to doubt, however, since substituted malondialdehydes are readily susceptible to continued oxidation by periodate[19].

A chain length of 20 was, however, supported by a methylation analysis on laminarin from L. hyperborea which gave, in addition to 2,4,6-tri-o-methyl glucose, tetramethyl glucose (5%), and a mixture of two di-o-methyl glucoses (8%). These were identified as 2,6 and 4,6 but it was demonstrated that, when 2,4,6-tri-o-methyl

glucose was subjected to the conditions used for hydrolysis of the methylated laminarin, 8% of a similar mixture of dimethylglucoses was produced. No structural significance was, therefore, attached to their presence in the hydrolysate of methylated laminarin.

Molecular weight determinations by physical methods were also reported. By a modification of Barger's method, a D.P. of 13-17 was obtained using a methylated sample, and osmometry, also on a methylated sample, indicated a D.P. of 17-25. Viscosity measurements gave a D.P. of 21 for an acetylated sample and a D.P. of 13 for a methylated sample. Measurement of reducing power by hypoiodite indicated one reducing group per 40 residues (cf. Barry[10] 36) and by bromine oxidation and titration of the resultant acid one in 48. These reducing powers were lower than would be expected on the basis of a linear molecule of ca. 20 glucose residues and thus there was uncertainty as to whether or not some branching was present. Periodate oxidation of unsubstituted laminarin, and measurement of the reduction of periodate and the release of formaldehyde and formic acid gave results which, after oxidation for 5 days at room temperature, were roughly consistent with a linear structure of D.P. ca. 20; but, as the oxidation slowly continued after this time, the results could not be

interpreted with confidence.

Percival and Ross[20] carried out a similar investigation on laminarin from L.digitata. This laminarin, known as soluble laminarin, is stated to require precipitation from aqueous solution with ethanol as the phenomenon of spontaneous deposition is not shown, in contrast to the behaviour of the previous sample from L.hyperborea. This corrects some previous confusion over solubilities (e.g. Krefting and Torup[3]; Barry[10]) possibly arising from the fact that the two species are superficially rather similar.

X-ray powder photography, specific rotation, specific viscosity, methylation analysis, and molecular weight determination by the modified Barger method and by periodate oxidation, all gave results which were not significantly different from those obtained for the insoluble laminarin from L.hyperborea. The only difference observed was the very low reducing power to hypoiodite, one reducing group per 112 residues being found. Some combined sulphate was present but, as fucose was detected in a hydrolysate, and the amount of both could be reduced by electrodialysis of a solution of the polysaccharide, contamination by fucoidin was indicated and sulphation of laminarin considered to be unlikely. It was noticed that the reducing power of

the laminarin increased as the fucoidin was removed but no explanation of this could be advanced.

Abdel-Akher, Hamilton and Smith[21] reduced insoluble laminarin with sodium borohydride and obtained a product, laminaritol, which showed very little reducing power to ferricyanide.

Syntheses of laminaribiose were reported[22,23] and its identity with the disaccharide isolated from laminarin was confirmed.

A partial acid hydrolysis was reported by Peat, Whelan and Lawley[24]. Three disaccharides were isolated and identified as laminaribiose, gentiobiose, and β,β-trehalose. The main component of the trisaccharide fraction was laminaritriose but four additional trisaccharides, each of which gave laminaribiose on partial acid hydrolysis, were obtained.

Three of these trisaccharides also gave gentiobiose and, presumably, are 3-O-β-gentiobiosyl glucose, 6-O-β-laminaribiosyl glucose and 3,6-diglucosyl glucose. This provides evidence in favour of a branched structure for laminarin. The fourth trisaccharide, which was non-reducing, gave β,β-trehalose which suggested that some of the polysaccharide chains might be terminated at the reducing end by a 1,1- linked β-glucose residue.

Friedlaender, Cook and Martin[25] investigated the

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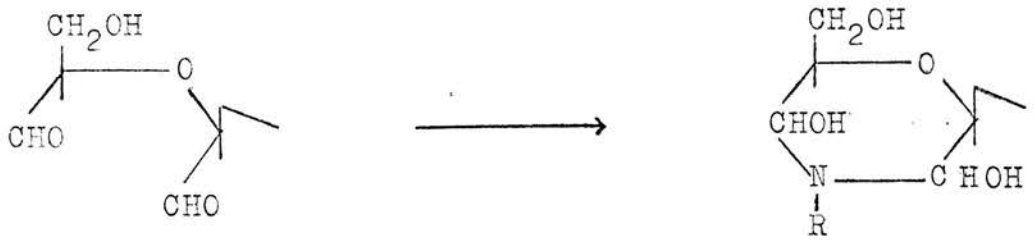

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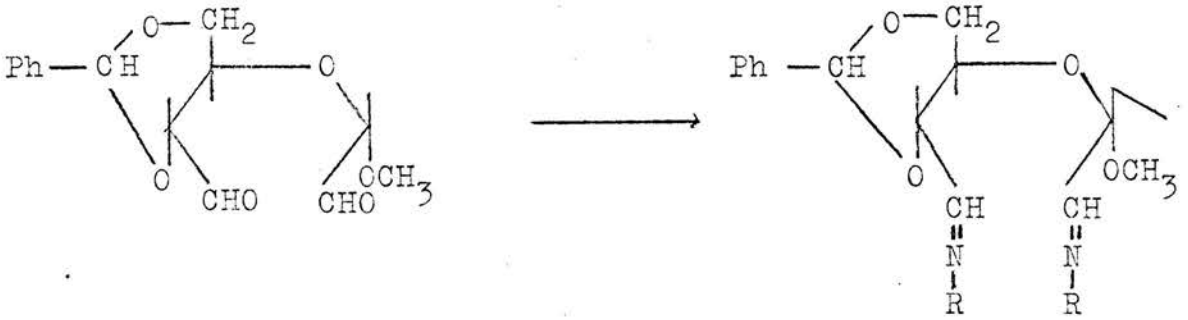

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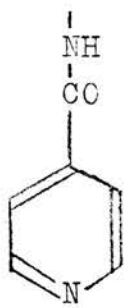


Fig. 3. ISONICOTINOYL HYDRAZINE DERIVATIVE OF

a) PERIODATE-OXIDISED LAMINARIN.

b) PERIODATE-OXIDISED METHYL 4,6-O-BENZYLIDENE-α-D-GLUCOSIDE.

molecular size and shape of laminarin by sedimentation and diffusion. Their results confirmed that the material was polydisperse and gave weight average D.P.s of 21 and 32 for insoluble and soluble laminarin samples respectively. Partially methylated samples were shown to have been degraded to material of lower D.P., thus casting doubt on most of the previous molecular weight determinations which were largely carried out on fully methylated samples. This degradation by dimethyl sulphate and alkali is not surprising in view of the susceptibility of laminarin to alkaline degradation. Corbett and Kenner[26,27] showed that laminarin was steadily degraded by oxygen-free lime water until ca. 50% degradation had occurred. They pointed out that the presence of one or more 1,6 linkages, as suggested by Peat et al.[24], could check this degradation.

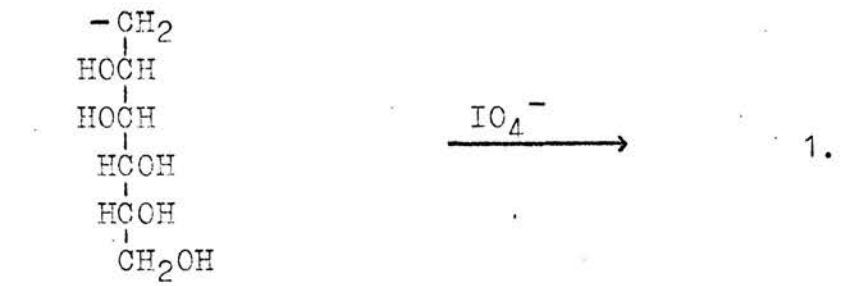
The isoniazid derivative of periodate-oxidised laminarin was prepared by Barry et al.[28]. The nitrogen content of 1.5% indicated a chain length of 16 assuming that the condensation with isoniazid occurred only at the non-reducing end group (Fig. 3a). Some doubt has, however, been cast on Barry's structure by Colbran et al.[29], who could prepare only the dialdehyde bis-isonicotinoyl hydrazone from periodate-oxidised methyl 4,6-O-benzylidene-α-D-glucoside (Fig. 3b).

The proportion of reducing end groups, on the other hand, was estimated[30] using the cyanohydrin reaction followed by the estimation of ammonia released on saponification. One reducing group per 25 residues was found, which was a rather larger proportion than that found previously by hypoiodite and bromine oxidations.

The identification of mannitol as a constituent of laminarin was first reported by Peat et al.[31,32]. The product of partial acid hydrolysis previously reported[24] as β,β-trehalose was shown to be, in fact, 1-O-β-glucosyl mannitol, and the non-reducing trisaccharide was similarly shown to be 1-O-β-laminaribiosyl mannitol. The glucosyl mannitol was stated to be identical with that which Lindberg isolated[33] from Fucus vesiculosus and subsequently synthesised[34]. Free mannitol was found in the monosaccharide fraction of the partial acid hydrolysate. Estimates of the ratio of mannitol to glucose in insoluble and soluble samples of laminarin gave one to 57 and one to 37 respectively. It was, therefore, established that a proportion of the otherwise reducing ends were terminated by mannitol, thereby accounting for the previously unexplained[18] low reducing power.

The quantity of formaldehyde liberated on over-oxidation by periodate was found[35] to be 0.5 moles per anhydrohexose unit. This result is in good agreement

Release of formaldehyde (mol.)



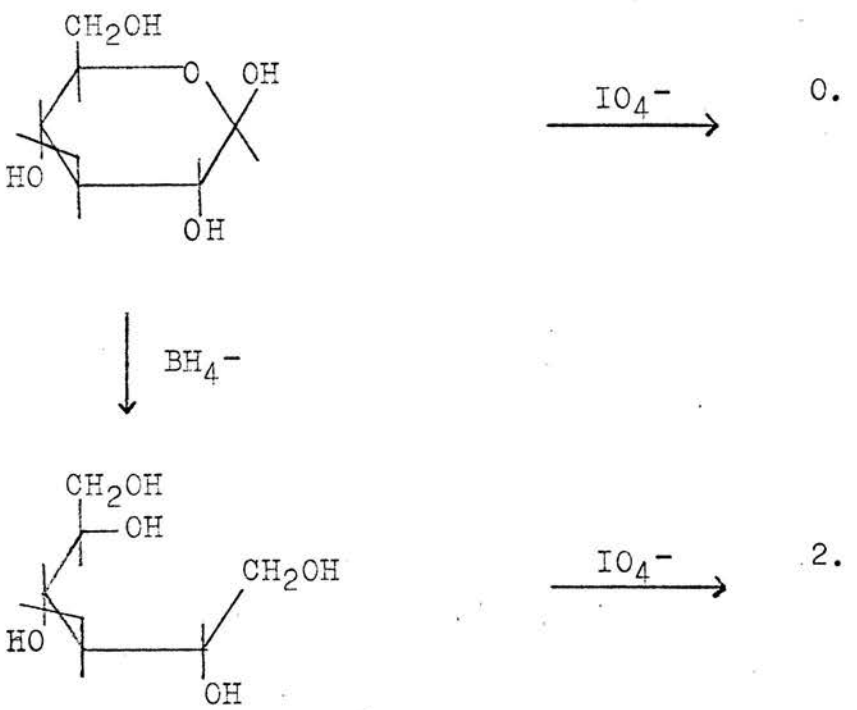


Fig. 4. PERIODATE OXIDATION OF LAMINARIN BEFORE AND AFTER BOROHYDRIDE REDUCTION.

with the results of the alkaline degradation experiments of Corbett and Kenner.

Unequivocal evidence for branching was produced by Broatch and Greenwood[36]. A sample of methylated laminarin, provided by Dr.A.G.Ross, which had been fractionated by precipitation with petroleum ether to remove low molecular weight material (ca. 20%), was subjected to molecular weight determination by isothermal distillation. The D.P. of 58 which was found was about three times the chain length as determined by the proportion of tetramethyl glucose, indicating, on average, two branch points per molecule. A similar result was obtained using laminarin which had been degraded by lime-water before methylation. Comparison of unmethylated laminarin with unmethylated, lime-water degraded laminarin in the ultracentrifuge indicated that the former was highly polydisperse and that the treatment with lime-water had preferentially degraded the lower molecular weight material present.

The proportion of molecules terminated by mannitol was estimated to be 30% by Unrau and Smith[37] who measured the release of formaldehyde on oxidation by periodate before and after reduction of the laminarin with sodium borohydride (Fig. 4). This information showed that the D.P. of 25 found by the cyanohydrin

method should be modified to a D.P. of 17 for the molecules terminated by a reducing glucose residue. The D.P. of whole laminarin was also stated to be 17; it therefore followed that the average D.P. of the reducing (G-chains) and the non-reducing molecules (M-chains) was similar. It was observed that laminarin was resolved into two components by electrophoresis in alkali, while homogeneity was displayed[38] if the laminarin was first reduced with sodium borohydride.

On the basis that one mole of formaldehyde was liberated from either a reducing glucose or a mannitol-terminal residue, and that two moles of formaldehyde were liberated from a 3-O- substituted sorbitol residue on oxidation by periodate, soluble laminarin was shown by Anderson, Hirst and Manners[39] to have a D.P. of 19 and to contain 75% of M-chains. Measurement of the release of formic acid during periodate oxidation gave an apparent chain length of 19. Corresponding figures for insoluble laminarin were 24 for chain length and D.P., and 46% of M-chains. The mannitol contents of the two samples were therefore one mole to 24 and 51 glucose residues respectively. These results were also consistent with the much lower reducing power of soluble laminarin. The release of 0.5 mole of formaldehyde on over-oxidation of insoluble laminarin, which was found by

Hough and Perry, was confirmed and it was suggested, on account of this, that any 1,6- linkages would be confined to the mannitol-terminated chains.

In 1958, Peat et al.[40] published full details of partial acid hydrolyses of laminarin, some of which had been reported earlier[24,31,32]. Many components were isolated from a hydrolysate of insoluble laminarin (137g.) and characterised.

Sugar	Yield (g).
D-Glucose	55
D-Mannitol	0.55
L-Fucose	0.55
Laminaribiose	25
1-O-β-Glucosyl mannitol	2.3
Gentiobiose	0.36
Isomaltose	0.09
Laminaritriose	20
1-O-β-Laminaribiosyl mannitol	1.1
3-O-β-Gentiobiosyl glucose	0.48
6-O-β-Laminaribiosyl glucose	0.36
3-O-β-Isomaltosyl glucose	0.12
1,6-di-O-β-Glucosyl mannitol	0.04
1-O-β-Isomaltosyl mannitol	0.01

In addition, laminaritetraose and laminaripentaose were isolated.

Thus three non-reducing trisaccharides were described instead of the one previously reported. Only

two reducing trisaccharides, giving rise to laminaribiose and gentiobiose on partial acid hydrolysis, were found, which contradicted the earlier suggestion that 3,6-di-O-β-glucosyl glucose had been obtained. There was, therefore, no positive evidence for branching.

Acid reversion experiments suggested that all three fragments containing the α-1,6- linkage could have arisen as artefacts to a sufficient extent to account for the quantities found in the hydrolysate. Structural significance was not ascribed to these compounds, or to the 1,6-di-O-β-glucosyl mannitol which was present also to a very small extent.

A sample of soluble laminarin was less extensively investigated; the main difference found was that this gave rise to a higher proportion of minor components i.e. contained more mannitol and 1,6- linkages. A larger proportion of fucose was also found together with traces of xylose and galactose. These sugars were ascribed to contaminating fucoidin. The mannitol contents of insoluble and soluble laminarin were found to be 1.7% and 2.7% respectively. Confirmation of the structures assigned to several of the isolated oligosaccharides was provided later by their synthesis[41].

Periodate oxidation studies by Anderson et al.[42] on insoluble laminarin before and after reduction with

borohydride indicated a D.P. of 24, that 46% of the chains were terminated by mannitol which constituted 1.9% of the polysaccharide and that the proportion of reducing glucose terminal residues was one in 45. The chain length was found to be 23 but the agreement between this figure and that for the D.P. is inconsistent with branching and indicated some inaccuracy in these figures.

Methylation studies on the sample with D.P. of 58 referred to previously[36] were also reported. The proportion of tetramethyl glucose gave a chain length of 23, and absence of 2,3,4-tri-O-methyl glucose showed that all the 1,6- linkages must be present as inter-chain rather than inter-residue linkages; a fact which was consistent with the identification of the main dimethyl glucose component as 2,4-di-O-methyl glucose. The lime-water degraded laminarin, which was shown to contain mannitol, gave a similar pattern on methylation analysis, the chain length of 19 being somewhat lower.

A sample of laminarin was reduced with potassium borohydride. The product, laminaritol, was unaffected by lime-water and a D.P. of 24 was obtained by measurement of the release of formaldehyde on periodate oxidation. Methylation of laminaritol gave material of D.P. 19 (by isothermal distillation) after removal of

low molecular weight material (6%), showing that much less degradation had occurred during methylation of this material.

The Barry degradation of laminarin was investigated by Hirst, O'Donnell and Percival[43] who demonstrated that after repeated degradations the product was largely non-dialysable. This supports the view that the 1,6-linkages are involved in inter-chain branching. If they were present as inter-residue linkages, the molecule would be cleaved at those points producing smaller fragments probably of dialysable size.

The picture of laminarin which emerges from this work so far is that of a heterogeneous material containing molecules with D.P.s of less than 20 to more than 60; the smaller molecules being linear β-1,3-linked chains and the larger molecules, which constitute most of the polysaccharide by weight, displaying a small degree of branching and carrying a terminal mannitol residue.

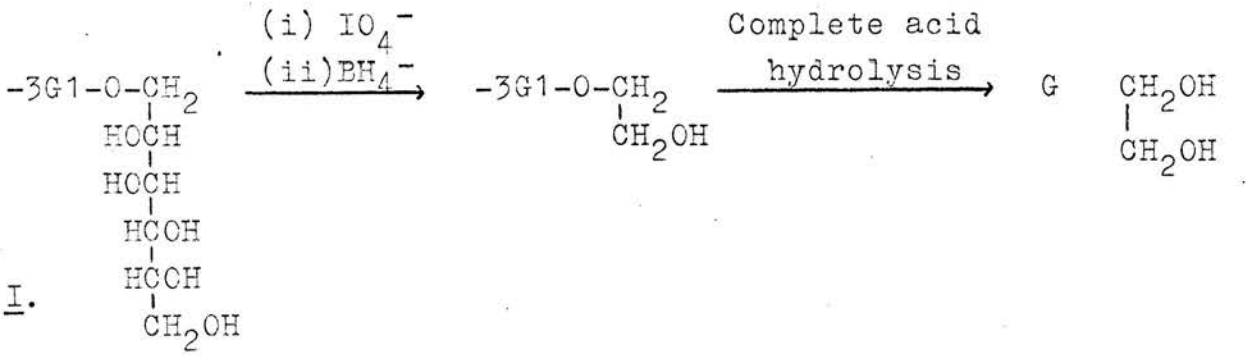
On the other hand, the structure of laminarin from Eisenia bicyclis appears[44] to differ markedly from this. The D.P. of 21 by cryoscopy approximated to the D.P. of 18 by hypoiodite oxidation, and mannitol was absent.

Oligosaccharides isolated by partial acetolysis included laminaribiose and gentiobiose in nearly equal

amounts. Higher oligosaccharides containing 1,3- and 1,6- linkages were tentatively identified up to laminaritetraose and gentiotetraose but the presence of 3,6-diglucosyl glucose was not claimed. Reduction of periodate by this laminarin was greater than that by laminarin of Laminaria species, and was consistent with the presence of 1,6- inter-residue linkages. It was also demonstrated that alkaline degradation ceased after the loss of one third of the material. Methylation studies showed the presence of tetramethyl glucose, 2,3,4- and 2,4,6-tri-O-methyl glucoses but no dimethyl glucose. The proportion of tetramethyl glucose was not stated but an unbranched structure was suggested by the other evidence.

Further work was, however, reported on laminarin from Laminaria species by Smith and his co-workers[45]. Bromine oxidation of laminarin which converted reducing glucose residues to gluconic acid residues enabled the separation, by ion exchange chromatography, of laminarin into mannitol-containing and mannitol-free fractions. The D.P. of the acidic fraction, by titration and by formaldehyde release on periodate oxidation, was about 16. A D.P. of 30 was obtained for the other fraction by estimation of the mannitol content. This conclusion contradicts the previous report[37] from this laboratory.

(a).



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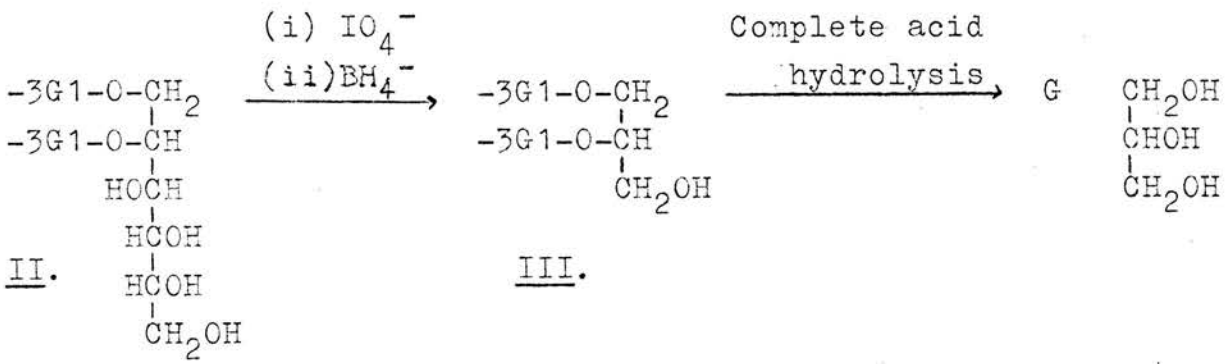


II. III.

Fig. 5. DEGRADATION BY PERIODATE OXIDATION AND BOROHYDRIDE REDUCTION OF:

a) 1-SUBSTITUTED MANNITOL RESIDUE

b) 1,2-DISUBSTITUTED MANNITOL RESIDUE.

The mannitol-containing fraction was subjected to periodate oxidation, reduction with borohydride and acid hydrolysis. Structure I(Fig. 5a) which was suggested by Peat et al., would give rise to ethylene glycol under these conditions, but this was not detected. Smith and his co-workers were therefore led to suggest that the mannitol residue was 1,2- disubstituted. (structure II; Fig. 5b). The alternative 1,3- disubstitution was excluded as erythritol was not formed by the above series of reactions.

Furthermore, assuming D.P.s of 16 and 30 for the mannitol-free and mannitol-containing fractions respectively, the proportion of glycerol released on oxidation, reduction and hydrolysis of whole laminarin may be estimated, the glycerol arising from the oxidation of mannitol residues (Fig. 5b) and terminal non-reducing glucose residues. (Fig. 6a). The quantity of glycerol found was greater than could be expected, on the basis of linear chains of 1,3- linked glucose residues, and therefore indicated the presence of branching, or 1,6- inter-residue linkages. (Fig. 6b).

This point was further investigated[46] by subjecting each of the two components of laminarin to the series of reactions; oxidation, reduction and mild acid hydrolysis to cleave acetal but not glucosidic linkages, followed

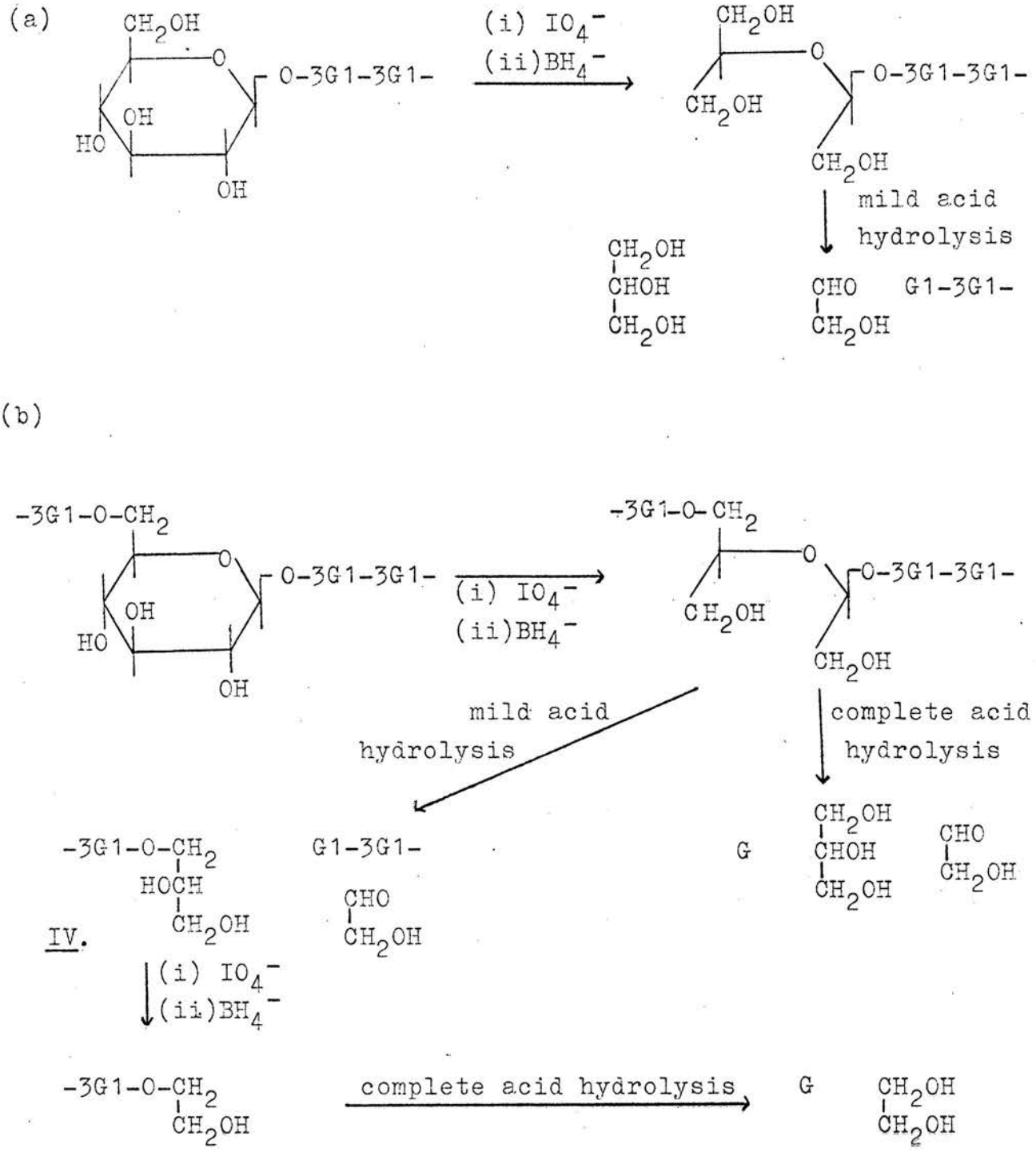


Fig. 6. DEGRADATION BY PERIODATE OXIDATION AND BOROHYDRIDE REDUCTION OF

(a) NON-REDUCING TERMINAL GLUCOSE RESIDUE

(b) 1,6-LINKED GLUCOSE RESIDUE.

by oxidation, reduction and complete acid hydrolysis. The isolation of ethylene glycol from both of the components after the completion of this procedure provided evidence for 1,6- inter-residue linkages. (Fig. 6b). The quantity of ethylene glycol isolated indicated one such linkage per G-chain and two such linkages per M-chain. Combined glycerol was also observed after the mannitol-containing fraction had been oxidised, reduced and subjected to mild acid hydrolysis. This would arise from 1,2- disubstituted mannitol (structure III; Fig. 5b) and from 1,6- inter-residue linkages. (structure IV; Fig.6b). It was not, however, stated whether the quantity found was consistent with the proposed molecular structure.

Support for 1,6- inter-residue linkages has been provided by methylation studies by Beattie, Hirst and Percival[47]. From a hydrolysate of methylated laminarin they isolated; tetramethyl glucose, one part; 2,3,4-tri-O-methyl glucose, one part; 2,4,6-tri-O-methyl glucose, 15 parts; and dimethyl glucose, 0.6 parts. The reported presence of 2,3,4-tri-O-methyl glucose indicated 1,6- inter-residue linkages in the polysaccharide.

The presence of mannose was first reported by Smith and Unrau[48] who detected it chromatographically in

hydrolysates of both components of laminarin. The ratio of mannose to glucose in a degraded mannitol-free fraction was found to be one to seven, and mannose was isolated from this material and characterised. On the other hand, Beattie et al.[47] found no mannose in their work.

More recently, the presence of mannose in a few enzymic hydrolysates of laminarin has been reported by Chesters and Bull[49], who did not, however, attempt rigorous characterisation. They obtained, also from an enzymic hydrolysate, a compound tentatively identified as 3,6-diglucosyl glucose but, while this is consistent with a branched structure for laminarin, its formation by enzymic transglucosylation could not be excluded.

There is therefore uncertainty concerning:

1. The presence of mannose as a possible constituent.
2. The mode of linkage of the mannitol residue.
3. The presence of 1,6- inter-residue or inter-chain linkages.

The experimental work reported in this thesis has been directed towards the solution of these three structural problems.

EXPERIMENTAL METHODS.

Aqueous solutions were concentrated in a rotary film evaporator using a bath temperature of less than 40° unless otherwise stated. Solvents were purified and dried where necessary by the methods given by Vogel.[50]

Drying of samples.

Before quantitative experiments samples were "dried" [51] to constant weight under reduced pressure at 100° over phosphorus pentoxide. Heating for one hour was generally sufficient. The dry weight content of seaweed samples was determined as indicated by Black.[52]

Determination of ash content.

Samples of carbohydrates were treated with a few drops of "Analar" sulphuric acid and heated to constant weight in a porcelain crucible. It was verified that sulphuric acid alone left no residue. To obtain ash contents of seaweed samples comparable to those obtained by Black[52] the sulphuric acid was omitted. It was found necessary to use a furnace temperature of 550° [53] to avoid volatilisation of the residue.

Estimation of sugars.

a) Reducing power was estimated by the copper reagent of Somogyi[54] using the iodometric procedure. The concentration of standard sugar solutions (ca. 10%) in saturated aqueous benzoic acid was determined polarimetrically, after allowing mutarotation to proceed to equilibrium, and suitable dilutions were then made for calibration of the reagent. Using a heating time of 20 min., glucose and mannose, up to concentrations of 2mg. per 5 ml. and 1.5 mg. per 5 ml., respectively, gave identical linear calibration graphs.

b) Total hexose content, whether combined or not, was determined by the method of Dubois et al.[55] Using sugar solution (1 ml.), phenol solution (2 ml; 5%) and sulphuric acid (5 ml; "Analar"), reproducible linear calibration graphs were prepared for glucose and mannose (concentrations up to 0.1 mg. per ml.). It was found that additional amounts of benzoic acid did not affect the resultant optical density.

Acid hydrolysis of carbohydrates.

a) Complete acid hydrolysis of soluble carbohydrate material was effected by heating at 100° with sulphuric acid (1.5 N; 3 hr. or 2 N; 2 hr.). When determination[56] of the reducing power of the hydrolysate was required,

the concentration of carbohydrate used was ca. 0.1% in order to minimise losses by acid reversion.

Hydrolysis of methylated or other insoluble polysaccharides was facilitated by a pre-treatment with formic acid (90%). The carbohydrate (25 mg.) was heated at 100° in a sealed tube with formic acid (2-3 ml.) for 0.5 to 6 hr. to effect solution. The formic acid was subsequently removed by a column of Amberlite IR 45 OH^- resin or by evaporating the solution to dryness, and dissolving the residue in water and evaporating to dryness again. A subsequent hydrolysis with dilute sulphuric acid was then used to hydrolyse any formyl esters and to complete the hydrolysis. This procedure causes very little degradation or demethylation of methylated sugars[57]. The sulphuric acid was neutralised with barium carbonate prior to chromatographic examination of the hydrolysate. In addition, deionisation using Biodeminrolit resin saturated with carbon dioxide[58] was attempted but, in the present work, epimerisation of glucose to give traces of fructose was detected.[59] The resin was not, therefore, generally used.

b) Partial acid hydrolysis was carried out with sulphuric acid (0.2 N) for ca. 30 min. at 100°.

c) Mild acid hydrolysis to effect cleavage of acetal but not glucosidic linkages[60] was carried out by

acidifying an aqueous solution of the carbohydrate with dilute sulphuric acid to give a final concentration of 0.1 or 0.25 N. The reaction was allowed to proceed at room temperature for several hr. before the products were investigated.

Methanolysis.

Methanolic hydrogen chloride was prepared by passing dry hydrogen chloride into cooled methanol which had been dried using magnesium.[50] The increase in weight was measured and the solution diluted to the required strength with dry methanol.

Methyl glycosides of methylated sugars were prepared from methylated sugars and polysaccharides by heating at 100° in a sealed tube with methanolic 3% hydrogen chloride for ca. 6 hr. in the case of sugars and ca. 20 hr. in the case of polysaccharides.[61] The solutions were neutralised with silver carbonate before concentration.

Methyl glycosides of unmethylated sugars were prepared by refluxing the sugars with methanolic 3% hydrogen chloride for 6 hr., but heating for 48 hr. with 6% hydrogen chloride was required to dissolve unmethylated laminarin adequately. The products were examined directly by paper chromatography without

neutralisation and concentration of the solutions.

Paper Chromatography.

Chromatograms were prepared using Whatman no.1 or no.3MM paper, which was washed chromatographically with water before preparative or quantitative experiments. Almost invariably, one descending development was employed but use was occasionally made of ascending solvent flow and also multiple excursions of the solvent.[62]

Solvents.

The most useful solvent systems were:

	Composition (v/v)
Ethyl acetate/pyridine/water[63]	10/4/3
Ethyl acetate/pyridine /water[64]	5/2/7
n-Butanol/pyridine/water[65]	6/4/3
n-Butanol/ethanol/water[66]	4/1/5
n-Propanol/ethyl acetate/water[67]	7/1/2
Ethyl acetate/acetic acid/formic acid (90%) /water[68]	18/3/1/4
Methyl ethyl ketone/acetic acid/water[69] (saturated with boric acid)	9/1/1
Methyl ethyl ketone/water	10/1
Methyl ethyl ketone/water/ammonia(0.880)	200/17/1

These solvents will be referred to by their

volumetric compositions. Where a two phase system was formed, only the upper layer was used both in the trough and in the bottom of the tank.[65] Distances moved by compounds relative to the distance moved by the solvent front or by glucose will be referred to as R_F and R_G values respectively. A preliminary experiment, suggested by the work of Durso and Mueller,[70] was carried out with a view to improving the efficiency of solvent 9/1/1. Compositions of 9/2/2, 15/1/3, and 15/4/2 were tried. No indication was found, however, of an increase in the development rate which did not involve marked reduction in the resolving power, and the experiment was not further pursued.

Spray Reagents.

Reagents used to reveal the position of compounds on chromatograms were:

1) Silver nitrate.

Both the method of Hough[71] and that of Trevelyan et al.[72] were used. Using Hough's method it was necessary to heat the sprayed paper for ca. 10 mins, while in the latter method a much shorter heating period, after the application of the ethanolic sodium hydroxide, was advantageous for the efficient revelation of some compounds, eg. methyl-α-glucoside. After both methods

the paper was washed with sodium thiosulphate[73] solution (ca. 10%) in place of ammonia solution.

2) Aniline hydrogen phthalate.[74]
3) Periodate / permanganate.[75]
4) Periodate / benzidine.[76]
5) Resorcinol / hydrochloric acid.[77]

In quantitative work, elution of compounds from chromatograms by the procedure of Dubois et al.[55] was found to be satisfactory. Compounds obtained in preparative separations were eluted by supporting the paper strip vertically, the top edge being held between two horizontal glass rods, and feeding water dropwise onto the top edge from a burette. The eluate was collected and concentrated.

Paper electrophoresis.

A "Shandon" apparatus was used, similar to that described by Foster[78] but lacking provision for cooling. Whatman No.1 paper was used with borate buffer (0.1 M; pH 10). Using 350 volts (ca. 10 mA) about 45 min. was a sufficient time. Xylose or glucose was used as a reference marker and tetramethyl glucose as the "non-mobile" marker. R_M values were calculated by assigning values of one and zero to the distances

travelled by the reference marker and the tetramethyl glucose respectively.

Column chromatography.

a) Cellulose.

The procedure of packing dry, as described by Hough, Jones and Wadman,[79] was adopted. The column was then washed with water followed by the solvent system to be used. Samples for chromatography were dissolved in the same solvent system, modified sufficiently to effect solution.

b) Ion-exchange resin.

The procedure described by Jones and Wall[80] was used to fractionate monosaccharides.

Dowex 50w X 8 resin (200-400 mesh) was converted into the Ba^{++} form by washing with portions of hydrochloric acid (2N), followed by water until neutral, and barium chloride solution (2N). Batches were washed by decantation as the rate of flow through a column was very slow.

Samples were applied to the column in aqueous solution and elution was carried out with water.

c) Sephadex.

Columns were prepared as described by Flodin[81]. Samples were applied in aqueous solutions, water being

used for the subsequent elutions. It was necessary to wash the column thoroughly with water before use to remove traces of soluble carbohydrate material.

Gas-liquid partition chromatography.

Gas-liquid chromatograms were kindly prepared by Dr. G.O.Aspinall[82] and his co-workers using a "Pye Argon Chromatograph".

Periodate oxidation.

All periodate oxidations were executed in the dark.[83] Reactions were followed by measurement of the reduction of periodate and the production of formic acid and formaldehyde.

a) Reduction of periodate.

The spectrophotometric method of Aspinall and Ferrier[84] was applied to appropriately diluted solutions.

b) Production of formic acid.

Potentiometric titration[85] to pH 6.25 with barium hydroxide solution was used. Residual periodate was reduced with a large excess of ethylene glycol solution before titration. The effect of carbon dioxide was minimised by using deionised water in the preparation of solutions and by passing a stream of nitrogen through solutions during titration. In addition, where it was

desired to leave formyl esters intact throughout the titration, the solution was kept immersed in an ice bath.

c) Determination of formaldehyde.

The reaction of formaldehyde with chromotropic acid which has been investigated by MacFadyen[86] and Bricker and Johnson[87] was used in the present work. Conditions similar to those described by MacFadyen were used with a few modifications.

The reagent was prepared according to Frisell et al.[88] by dissolving the sodium salt of chromotropic acid (1g.) in water (100 ml.) and adding sulphuric acid (400 ml; 25N), the latter being prepared by the addition of sulphuric acid (278 ml.; "Analar") to water (160 ml.). This reagent was stable, in apparent contrast to that of O'Dea and Gibbons.[89] An adequate amount of sulphuric acid is provided by this reagent for a final concentration of 18-20 N during the development of the colour. Higher concentrations have been used for the determination of hexoses[90] and should therefore be avoided here. The reagent was calibrated against suitably diluted formalin which was standardised using iodine and thiosulphate.[91]

Residual periodate was reduced with sodium sulphite[92] before the addition of the chromotropic acid reagent. Bleaching of the purple colour by sulphite

has, however, been reported[92,93] while other workers[94,89] have found no interference. This was investigated by preparing the following solutions:

		A	B	C	D
Formaldehyde solution (28.2 µg/ml.)	(ml.)	0	10	10	10
Sodium metaperiodate solution(0.3M)	(ml.)	3	1	3	5
Water	(ml.)	12	4	2	0

Samples (0.5 ml.) from B,C and D were added to sodium sulphite solution (0.33 M, 1.00 M or 1.67 M; 0.5ml.) followed by chromotropic acid reagent (9 ml.). The solutions were heated at 100° for 30 min., allowed to cool and thiourea[88] solution (4.6%, 2 ml.) was added before determination of the optical densities at 570 mµ. The reagent blank, prepared simultaneously, consisted of a sample (0.5 ml.) from A treated with sulphite (1.00 M; 0.5 ml.) and then as above.

The observed optical densities were:

	Sodium sulphite concentration.(M)		
	0.33	1.00	1.67
B	0.425	0.418	0.406
C	0.432	0.428	0.421
D	-	0.442	0.439

These results are consistent with a slight bleaching action but, in view of the wide variation

covered (3.3 to 83.3 moles of sulphite per mole of periodate), the effect is not of much practical importance. Presumably a ratio of 4 moles of sulphite per mole of periodate is required as the sample of D treated with sulphite (0.33 M) behaved abnormally.

Residual oxopolysaccharide interfered by forming a brown colour (λ_{max} = 468 mμ) during the heating period in the presence of the chromotropic acid reagent. The ratio of the extinction coefficient at 570 mμ to that at 468 mμ was 0.57 in contrast to the ratio of 2.26 obtained for the purple formaldehyde-chromotropic acid colour. The formaldehyde concentration could therefore be estimated from the optical densities at 468 and 570 mμ.

The use of a blank determination containing the appropriate amount of unoxidised polysaccharide was unsatisfactory as this appeared to produce a disproportionately strong brown colour. Throughout the present work, precipitation of polysaccharide material with ethanol[95] was used. This procedure almost completely removed the interference and generally gave low blank values.

A typical procedure for the oxidation of a laminarin sample was to treat the laminarin solution (ca. 0.4%; 10 ml.) with sodium metaperiodate solution (0.3 M; 0.5 ml.). The progress of the reaction was followed by placing samples (1 ml.) in sodium sulphite

solution (0.5 M; 0.5 ml.) and adding ethanol (4 ml.). The mixture was allowed to stand for one to two days at 2° before centrifuging. If the supernatant was turbid it was advantageous to stand the corked centrifuge tube at room temperature for several hours followed by a second centrifugation. A portion (1 ml.) of the supernatant was treated with the chromotropic acid reagent and thiourea solution as before, the optical density being then determined using 2cm. cells.

A reagent blank was prepared by using water (10 ml.) in place of polysaccharide solution. A blank containing unoxidised polysaccharide could be prepared by the addition of sodium sulphite solution (0.5 M; 5.25 ml.) to the periodate solution (0.3 M; 0.5 ml.) before addition of the polysaccharide solution (10 ml.); samples of 1.5 ml. being withdrawn from this solution for estimation. As occasional slight variations occurred, greater accuracy was obtained by including one or two standard solutions of formaldehyde with each batch of determinations rather than by using a previously prepared calibration graph.

MATERIALS

1-O-β-Laminaribiosyl mannitol and 1-O-β-laminaritetraosyl mannitol were kindly supplied by Dr.J.R.Turvey. Laminaribiose and 1-O-β-glucosyl mannitol were kindly supplied by Dr.D.H.Hutson.

D-Arabitol.

D-Arabitol was prepared by the reduction of D-arabinose with borohydride.[21] D-Arabinose (5 g.) in water (100 ml.) was treated with potassium borohydride (1 g.) in water (50 ml.). The optical rotation reached a constant value after 45 min. and the solution was then neutralised with glacial acetic acid and evaporated to dryness. The residue was acetylated with acetic anhydride (75 ml.) and concentrated sulphuric acid (5 ml.). The pentaacetate was recrystallised from water and had m.p. 75-6° and $[\alpha]_D$ +36.4° (c.0.8 in chloroform).(lit.[96] 74-5° and $[\alpha]_D$ +37.2°). Deacetylation in acetone with alcoholic potassium hydroxide did not however readily afford arabitol. The reduction was therefore repeated and the neutralised solution was deionised using a column of Amberlite IR 120 H^+ (12x1.7 cm.) and a column of Amberlite IRA 400 OH^- (10x3.6 cm.). The product (2.3 g.; 45%) was chromatographically pure and had m.p.

103-4° after crystallisation from ethanol (lit.[96] 103°). Further washing of the resin columns yielded very little more product (cf.[21] 40% yield of dulcitol from galactose).

Arabitol (300 mg.) was heated with benzoyl chloride (2 ml.) in pyridine (5 ml.) for 15min. at 100° and the solution was poured into cold 2N sodium carbonate (40 ml.). The arabitol pentabenzoate was extracted with chloroform and, after purification and recrystallisation from ethanol the product (760 mg.; 57%) had m.p. 151-2° and $[\alpha]_D$ +28° (c. 1.06 in chloroform). As physical constants for this compound have not been published, an analysis was obtained from Drs. Weiler and Strauss. Calculated for $C_{40}H_{32}O_{10}$: C, 71.4; H, 4.76. Found: C, 70.8; H, 4.58.

Cellobiitol.

Cellobiitol was similarly prepared by the addition of potassium borohydride (0.15 g.) in water (10 ml.) to a solution of cellobiose (1g.; 20 ml.). The rotation became constant after 2-3 hr. The neutralised solution was then passed through a column of Amberlite IR 120 H^+ (2.5 x 1.7 cm.) and a column of Amberlite IRA 400 OH^- (9 x 1.7 cm.). The deionised solution and column washings were combined (200 ml.) and evaporated to dryness. The residue was recrystallised from methanol

to give the product (0.35 g.; 35%) which had m.p. 98-101° (lit.[97] 106-106.5° for cellobiitol monohydrate). The cellobiitol was chromatographically pure and had R_G 0.41 in 10/4/3 (cf. R_G 0.52 for cellobiose).

Laminaribiitol.

Laminaribiose (400 mg.) in water (8 ml.) was treated with potassium borohydride solution (60 mg.; 4 ml.). A constant optical rotation was reached in 5 hr., apparently indicating that the reduction was more rapid than under the conditions of Bragg and Hough.[98] Deionisation was carried out with a cation exchange column (4 x 1 cm.) and an anion exchange column (4.5 x 1.7 cm.) as in the preparation of cellobiitol. The residue (130 mg.; 31%) obtained on evaporation of the solution was recrystallised from ethanol but only a small quantity of gelatinous material m.p. 124-5° was obtained. Chromatography showed that the product was contaminated with a trace of sorbitol. It was therefore purified by thick paper chromatography and recovered by freeze drying to give chromatographically pure laminaribiitol R_G 0.65 in 10/4/3 (cf. R_G 0.77 for laminaribiose).

Laminarin Samples.

The laminarin samples used in this work were:

1). Sample BB2, which was the sample of insoluble laminarin from L. hyperborea (=cloustoni) described by Anderson et al.[42]

2). Sample SL5, a sample of soluble laminarin prepared by Dr. W. A. P. Black from L. digitata.

3). A sample from the Liverpool Borax Company kindly supplied by Dr. A. T. Bull. This was used by Chesters and Bull[49] in their enzymic studies.

4). A sample kindly supplied by Professor F. Smith. The analysis reported by Professor F. Smith for this sample was glucose (84.8%), mannose (2.4%) and mannitol (12.8%).

5). A sample extracted from L. hyperborea which was harvested by Mr. E. Booth at Gairloch on 13th December, 1961. (See Appendix A)

6). A sample extracted from L. saccharina harvested at St. Abbs on 15th October, 1962. (See Appendix A)

Determination of the ash content and the glucose content, the latter by cuprimetric titration of hydrolysate, was carried out to check purity.

Sample	BB2	Borax	Smith	5	6
Ash (%)	0.6	1.5	-	1.2	0.8
Glucose content(%)	94	90	90	87	91

Sample 6 had $[\alpha]_D$ -15° (c. 1.5 in water)

$[\alpha]_D$ +2° (c. 0.7 in N sodium hydroxide).

These compare with figures reported by Anderson:[99]

Sample	BB2	SL5
Ash (%)	0.45	-
Glucose content (%)	94	94
$[\alpha]_D$ (water)	-9°	-14°
$[\alpha]_D$ (N sodium hydroxide)	+9°	-

PERIODATE OXIDATION OF MODEL COMPOUNDS

Discussion.

The 1,3 polymeric linkage in laminarin restricts oxidation by periodate to the terminal residues of the molecules. Measurement of the production of formic acid and formaldehyde, and of the reduction of periodate, gives information about these terminal residues, from which the molecular structure may be deduced. It is necessary, however, to establish conditions of concentration, pH, and temperature which lead to interpretable results.

The mechanism of even the simplest oxidation, that of a 1,2 glycol, may be first order or second order,[100] and the rate constants exhibit maxima and minima at various values of pH.[101] Oxidation of carbohydrates has been studied by many workers including Courtois,[102] Neumüller and Vasseur,[103] and Cantley, Hough and Pittet,[104] who have shown that reactions other than that of glycol fission are involved. Oxidation of activated methylene groups, hydrolysis of esters and hemiacetals and cyclisation of intermediates to form hemiacetals may occur. These hydrolyses and cyclisations do not involve the reduction of periodate and are acid-base catalysed. They may be minimised, therefore, by the use of a

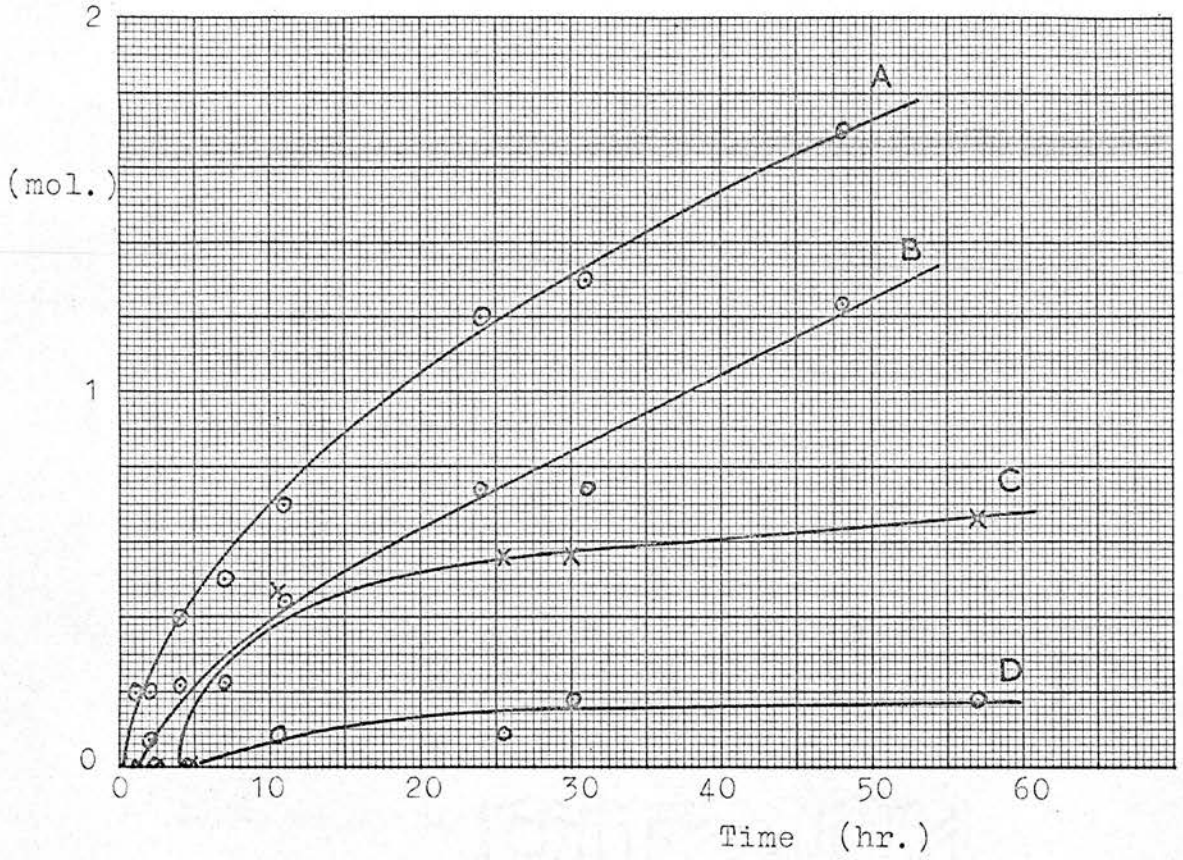
sufficiently high concentration[105] of periodate to complete the oxidation rapidly, and of pH ca. 4 which has a low catalytic activity. A suitable pH is obtained by oxidation in an unbuffered solution of sodium metaperiodate.

Model compounds have been oxidised under various conditions, some of which have been applied to laminarin. It was usually necessary to construct a progress curve which was extrapolated to zero time in order to estimate the initial extent of the reaction.

Reducing glucose terminal residue.

It has been shown by Clancy and Whelan[106] that the rate of oxidation of non-reducing terminal glucose residues is very slow if oxidation is conducted with a low concentration of periodate (0.4 mM). Oxidation of disaccharides under these conditions should therefore give information on the behaviour of the reducing residue alone. This reaction was investigated by measurement of the release of formic acid at 2° from glycerol, glucose, cellobiose, maltose and laminaribiose. The simultaneous reduction of periodate was also determined in the case of glycerol, glucose and cellobiose.

Owing to the difficulty of accurately titrating



0.4 mM Periodate at 2°.

A. Reduction of periodate by 0.065 mM cellobiose.

B. Release of formic acid from 0.065 mM cellobiose.

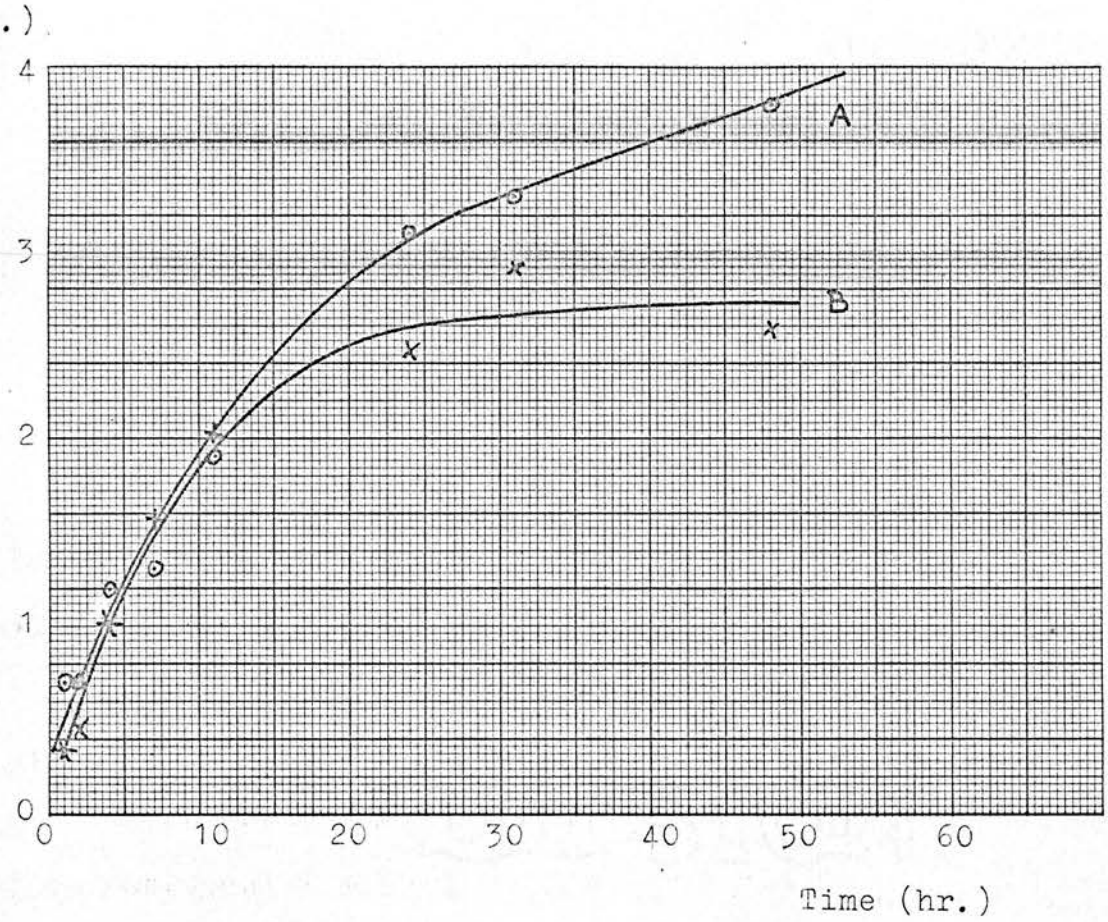
C. Release of formic acid from 0.052 mM maltose.

D. Release of formic acid from 0.054 mM laminaribiose.

Fig. 7. PERIODATE OXIDATION OF CELLOBIOSE, MALTOSE AND LAMINARIBIOSE.

10^{-4} to 10^{-5} N formic acid the results were slightly erratic but useful conclusions can nevertheless be drawn. The oxidation of 0.059 mM glycerol gave one mol. of formic acid and reduced two mol. of periodate as expected. The release of formic acid from laminaribiose was very slow, amouting to only 0.1 mol. after oxidation for 1-2 days (Fig.7). This could have arisen either from the hydrolysis of the formyl ester or from the oxidation of the non-reducing glucose residue, and is obviously consistent with the very slow oxidation of the latter.

Both maltose and cellobiose might be expected readily to give one mol. of formic acid under these conditions. This was not found, the release of formic acid being slow in both cases (Fig.7). Furthermore, although these compounds differ only in the configuration of the glucosidic linkage, an apparent difference existed between the rates of release of formic acid. Clancy and Whelan[106] have previously shown that 3-O-β- and 4-O-β- glucosyl sorbitol behaved differently under these conditions. Comparison of the release of formic acid with the reduction of periodate (Fig.7) for cellobiose indicated that the former was unexpectedly high. Hydrolysis of the formyl ester is unlikely, and it seems that the sugar must be reacting partly in the



0.043 mM Glucose with 0.4 mM periodate at 2°.

A. Reduction of periodate.

B. Release of formic acid.

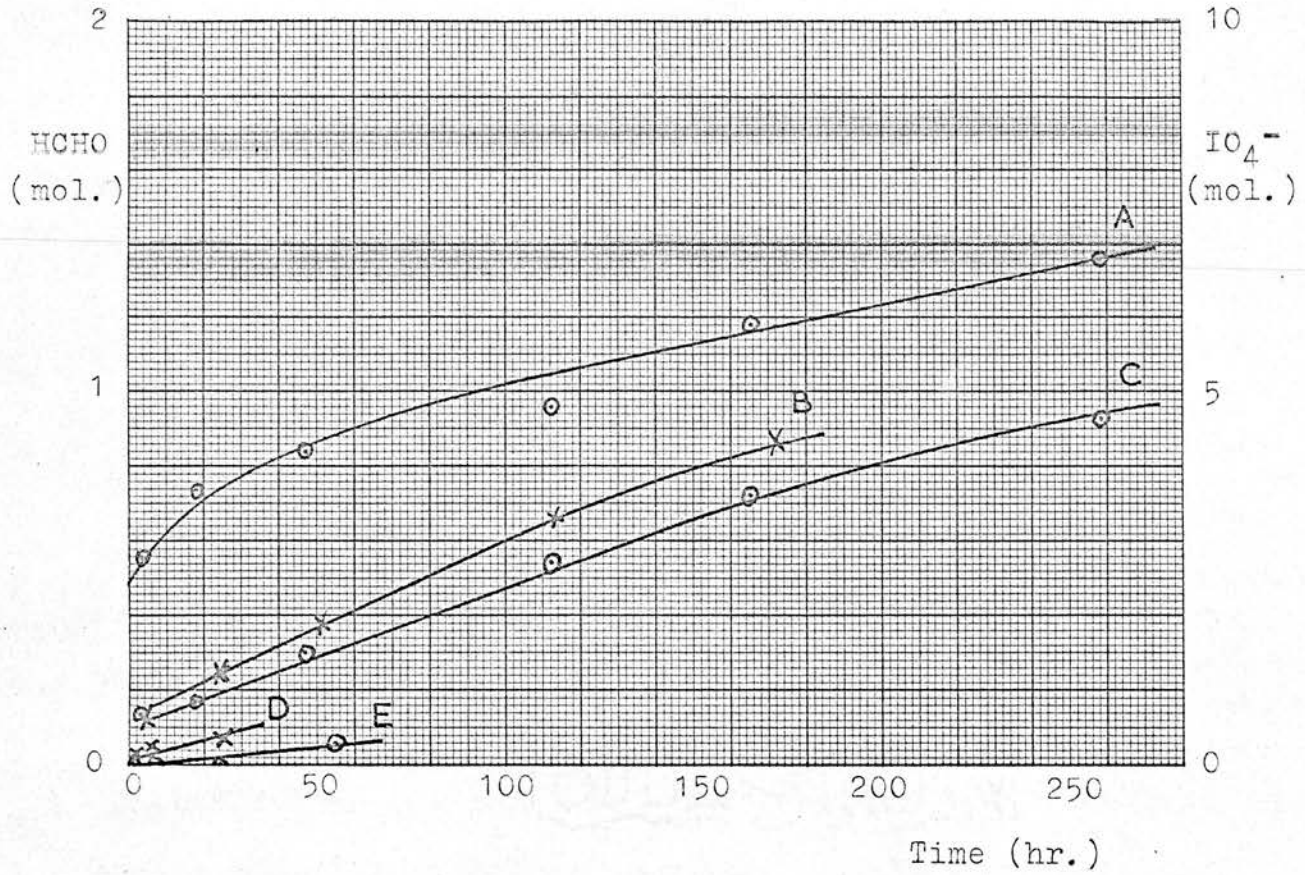
Fig. 8. PERIODATE OXIDATION OF GLUCOSE.

aldehydo- form.

The oxidation of glucose also took an unexpected course in that the release of formic acid was indistinguishable from the reduction of periodate up to two molar proportions (Fig.8). Initial oxidation in the aldehydo- form appears to be required by these facts. Hughes and Nevell,[107] who used more concentrated solutions (12.5 mM glucose; 87.5 mM sodium metaperiodate), also found a coincidence between the yield of formic acid and the reduction of periodate but their titration of formic acid to the phenolphthalein end-point (pH 9) will presumably have involved hydrolysis of the formyl ester. Warsi and Whelan[108] have concluded that glucose was oxidised (20 mM glucose; 200 mM sodium metaperiodate) in the cyclic form with the production of a formyl ester.

While there is ample evidence that reducing sugars are oxidised normally as cyclic structures, resulting in the intervention of formyl esters, this theory does not apparently fit the results of some of the very slow oxidations in dilute solution which have been described here.

Oxidation of reducing terminal residues has also been investigated by the determination of formaldehyde release on oxidation with more customary concentrations of periodate (ca. 15 mM). The release of formaldehyde



14.3 mM Periodate.

A. Reduction of periodate by 1.15 mM laminaribiose at 18°.

B. Release of formaldehyde from 0.92 mM laminaritriose at 18°.

C. Release of formaldehyde from 1.15 mM laminaribiose at 18°.

D. Release of formaldehyde from 0.91 mM maltose at 2°.

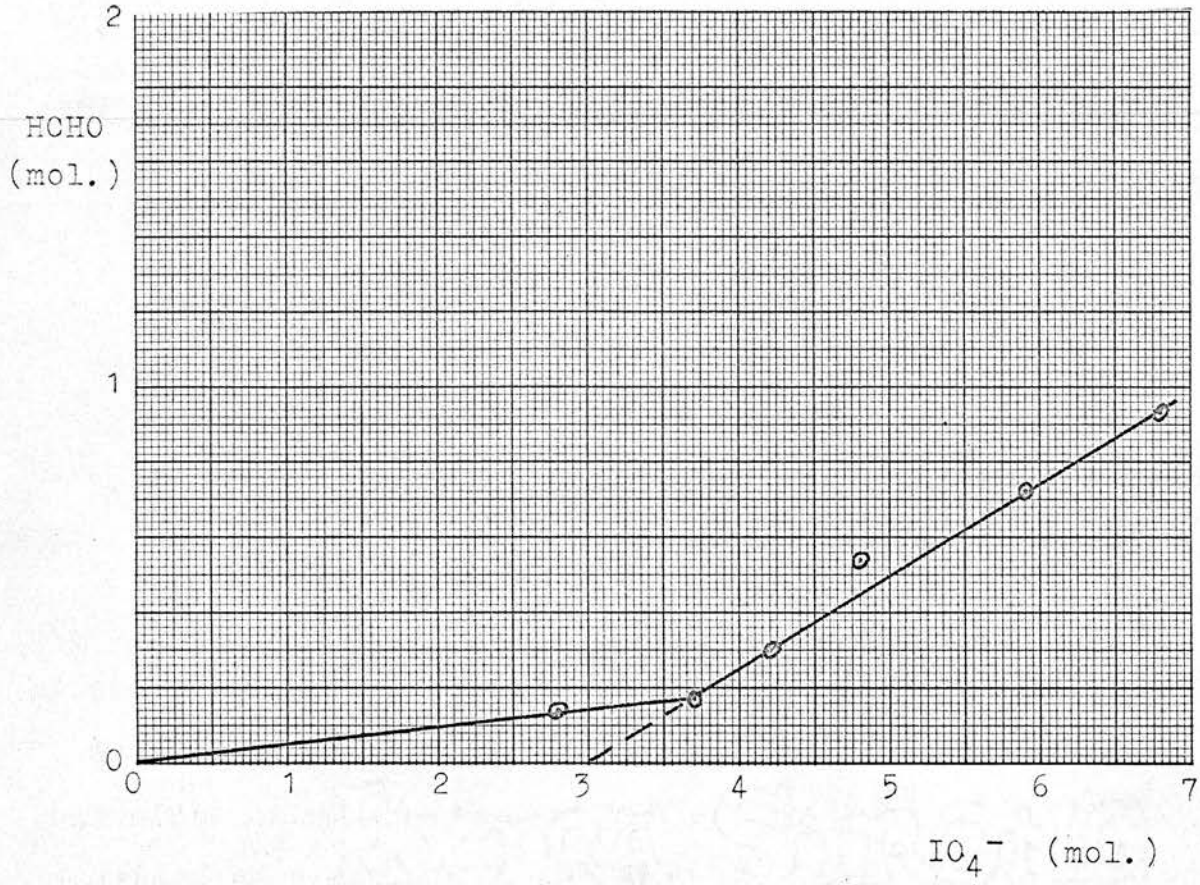
E. Release of formaldehyde from 1.29 mM laminaribiose at 2°.

Fig. 9. PERIODATE OXIDATION OF MALTOSE, LAMINARIBIOSE AND LAMINARITRIOSE.

at 2° from maltose and laminaribiose was found to be very slow, none being detected from the latter until after two days (Fig.9). This confirmed the results of Anderson et al.[42]

The release of formaldehyde at room temperature from both laminaribiose and laminaritriose was determined and the reduction of periodate by the laminaribiose determined simultaneously (Fig.9). After three to four days, about 0.5 mol. of formaldehyde was liberated from both compounds. Oxidation of laminaribiose was continued for eleven days when the yield of formaldehyde approached one mol. It should perhaps be mentioned that these rates are very different from the findings of Schiffman, Kabat and Leskowitz[109] who had to invoke oxidation of the acyclic form to explain their results for the oxidation of laminaribiose. In view of the small scale of their experiments, it is considered that the present results are the more reliable.

Head and Hughes[110] achieved complete oxidation of cellobiose in twenty days at 20°, the release of formaldehyde and the reduction of periodate rising to 2 and 11 molar proportions respectively. A plot of the release of formaldehyde against reduction of periodate showed the expected initial reduction of four mol. of periodate followed by a linear production of formaldehyde



1.15 mM Laminaribiose with 14.3mM periodate at 18°.

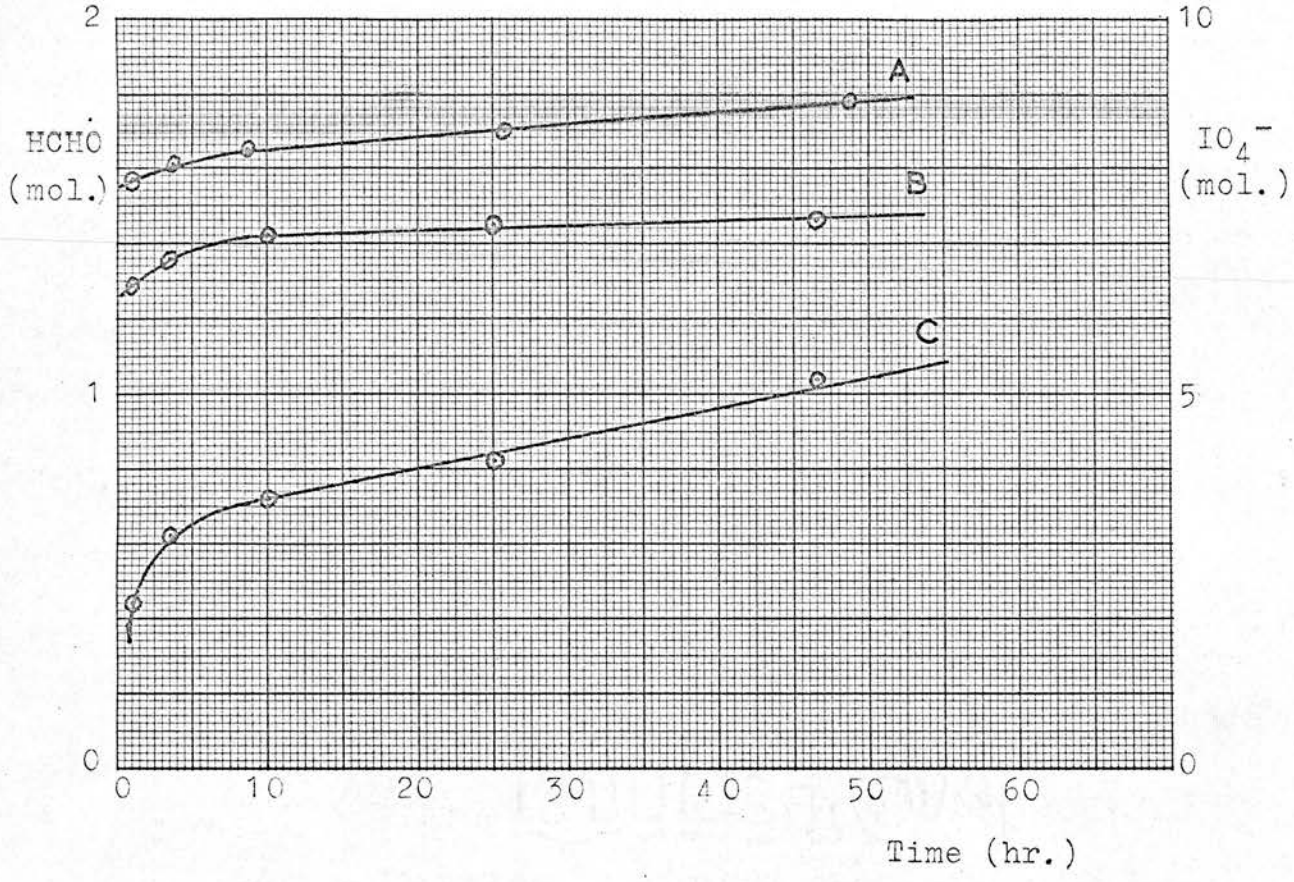
Fig. 10. PERIODATE OXIDATION OF LAMINARIBIOSE.

up to two mol. A plot for laminaribiose gave a similar result, the initial reduction of three mol. of periodate being observed (Fig.10).

It appears therefore, that on the hydrolysis of the formyl ester, overoxidation proceeds smoothly and no definite stage exists when the proportion of terminal reducing residues can be estimated by the release of formaldehyde. The estimation has been made however by Lawley[111] who showed that the rate of reduction of periodate by laminaridextrins during overoxidation was directly proportional to the content of terminal reducing residues. He determined the rate of over-oxidation of laminarin and, provided that the overoxidation did not proceed as far as the end of any molecule or as far as any 1,6- inter-residue linkages, a valid estimate of the proportion of reducing end group would be obtained. Parrish and Whelan[112] have measured the D.P. of synthetic amyloses by following the rate of release of formaldehyde on overoxidation, using maltodextrins as reference substances.

Sorbitol terminal residue.

In an attempt to estimate the reducing glucose terminal residues, Unrau and Smith[37] used borohydride reduction to replace these by sorbitol terminal residues.



0.81 mM Laminaribiitol at 2°.

A. Release of formaldehyde with 57 mM periodate.

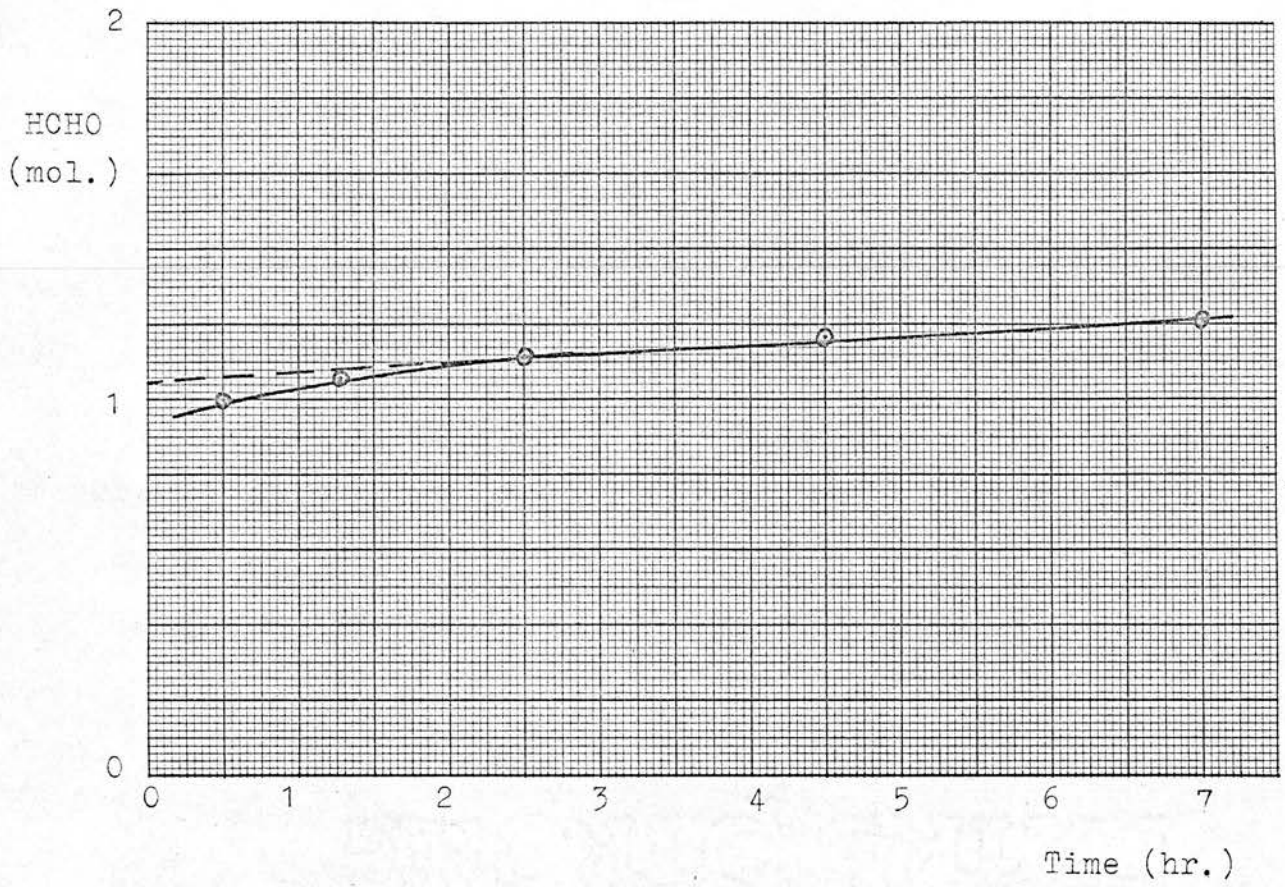
B. Release of formaldehyde with 14.3 mM periodate.

C. Reduction of periodate with 14.3 mM periodate.

Fig. 11. PERIODATE OXIDATION OF LAMINARIBIITOL.

It was stated that the latter provided two molar proportions of formaldehyde on oxidation. Cantley et al.[104] found this to be the case for 4-O-β-glucosyl sorbitol but not for 3-O-methyl sorbitol which yielded only 1.5 mol. Laminaribiitol was, therefore, oxidised in unbuffered solution at 2° to retard overoxidation (Fig. 11). The immediate release of formaldehyde amounted to only 1.4 molar proportions, the reduction of periodate also being rather low. The experiment was repeated using a four-fold increase in periodate concentration to reduce the extent of hemiacetal formation[105] but the immediate release of formaldehyde was raised only to 1.6 molar proportions.

The alternative approach of encouraging side reactions was, however, successful (Fig. 12). The use of one tenth of the original periodate concentration reduced the yield of formaldehyde to 1.0 mol., the consumption of periodate being about one mol. less than that originally found. Room temperature was used because negligible overoxidation would take place in the shorter time required for this reaction. The result agrees well with that obtained by Clancy and Whelan[106] who showed further that the second molar proportion of formaldehyde was obtained much more readily from cellobiitol than from laminaribiitol.



Release of formaldehyde from 0.448 mM laminaribiitol with 1.43 mM periodate at 18°.

Fig. 12. PERIODATE OXIDATION OF LAMINARIBIITOL.

The oxidation in dilute periodate solution of laminaritol should therefore result in the liberation of only one molar proportion of formaldehyde from each original reducing glucose residue.

Non-reducing glucose terminal residue.

Anderson et al.[42] obtained readily one mol. of formic acid from laminaribiose on oxidation in unbuffered solution at 2°. It has been shown, and confirmed here, that formaldehyde is not detected until after two days, which indicated that the formyl ester is reasonably stable under these conditions. The formic acid was therefore derived from the non-reducing glucose moiety. It has also been confirmed here (p.41) that the release of formic acid from non-reducing glucose terminal residues on oxidation in dilute solution at 2° must be very slow.

It may be noted that if any 1,6-linked glucose residues are present in the laminarin molecule their behaviour might be expected to be indistinguishable from that of non-reducing glucose terminal residues.

Mannitol terminal residue.

Oxidation of mannitol (0.35 mM mannitol; 60 mM periodate) in acetate buffer (pH 3.5) at room temperature

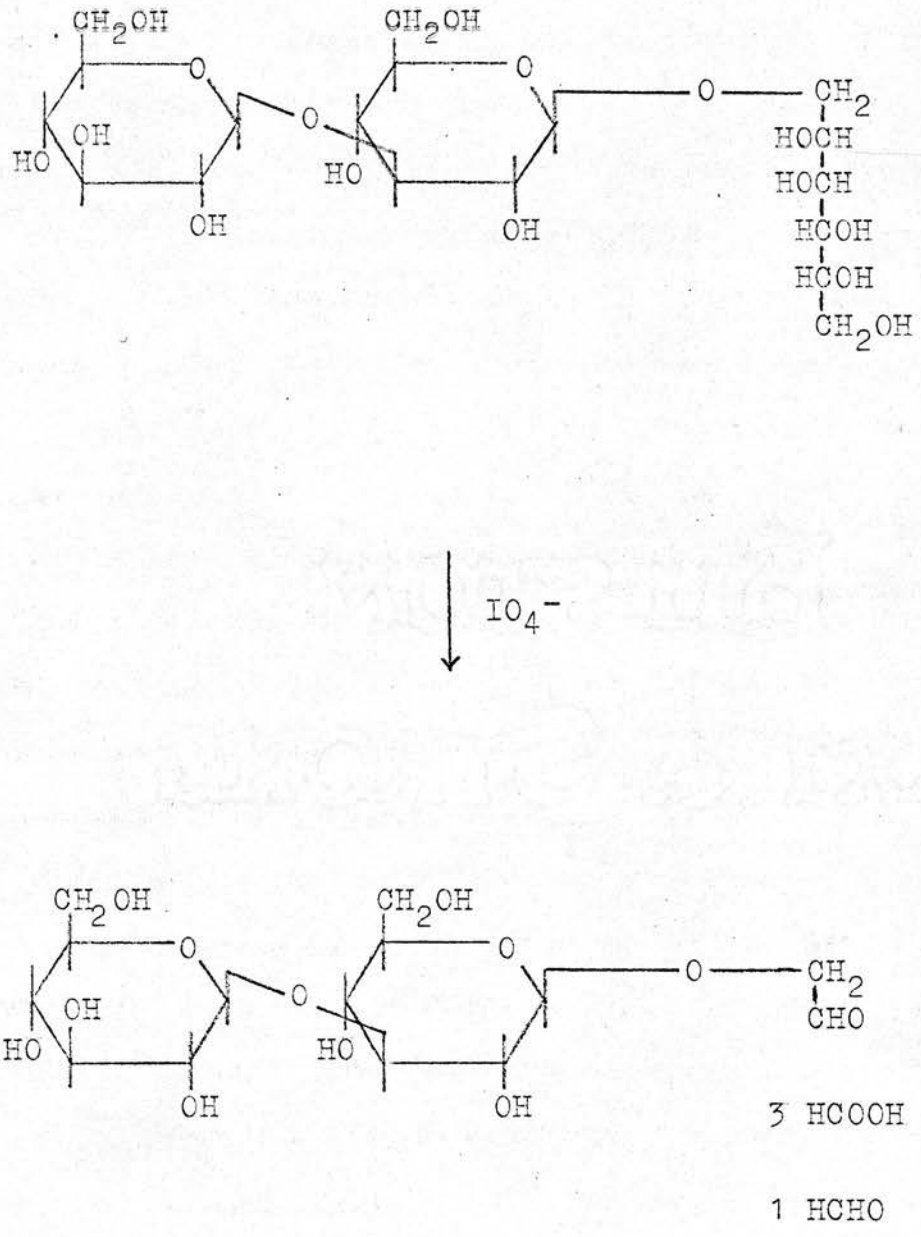


Fig. 13. PERIODATE OXIDATION IN DILUTE SOLUTION OF 1-O-β-LAMINARIBIOSYL MANNITOL.

gave an initial release of 1.8 mol. of formaldehyde. The discrepancy between this and the theoretical yield of 2.0 molar proportions can be ascribed[113,114] to the participation of intermediate oxidation products in hemiacetal formation. Unsubstituted mannitol is not, however, a very suitable model compound.

Oxidation at 2° in unbuffered solution of 1-O-β-laminaribiosyl mannitol (1.2 mM laminaribiosyl mannitol; 14.3 mM periodate) immediately gave a virtually quantitative yield of formaldehyde. Oxidation of laminarin under these conditions should, therefore, release one mol. of formaldehyde per mannitol residue.

A yield of 2.9 mol. of formic acid was obtained from this sample of laminaribiosyl mannitol on oxidation in dilute solution (0.055 mM laminaribiosyl mannitol; 0.4 mM periodate) at 2°. It has been established that formic acid is not released from the non-reducing glucose terminal residue under these conditions, showing that the formic acid arose from the complete oxidation of the mannitol residue to a stable glucosidically linked acetaldehyde derivative (Fig. 13).

This work indicates that the D.P. of laminarin could be estimated by the oxidation of laminaritol in dilute solution. The release of one mol. of formaldehyde would be expected from both sorbitol and mannitol

terminal residues, thus giving the proportion of these present in the sample.

Experimental.

Standardisation of dilute alkali.

The reservoir of an automatic filling burette was filled with deionised water (ca. 500 ml.), and a stream of carbon dioxide-free air passed through to remove all carbon dioxide from the system. Saturated barium hydroxide (ca. 1 ml.) was added and the solution thoroughly mixed.

A standard solution of 0.00153 N benzoic acid was prepared by weighing, and portions (5 ml.), diluted with deionised water (ca. 20 ml.), were titrated to pH 7.3. A stream of nitrogen was passed through the solution during titration. Titration of portions (25 ml.) of deionised water to the same pH required ca. 0.7 ml. and this blank value was subtracted from the benzoic acid titres. The alkali was found to be 0.00069 N.

The standardisation was repeated in a similar manner using hydrochloric acid which had been previously standardised at ca. 0.1 N strength against sodium carbonate and subsequently diluted by a factor of 100. Using pH 7 as end-point, the normality of the alkali was

found to be 0.00071 N, in reasonable agreement with the previous value. It was found that the pH of solutions during titration decreased gradually with time. The rate at which titrations were completed was, therefore, kept reasonably uniform.

Reduction of periodate and release of formic acid by glycerol, glucose and cellobiose in dilute solution at 2°.

A 2.78% solution of glycerol was prepared by weighing. The strength of this solution was checked by a preliminary oxidation followed by estimation of formaldehyde. A portion (1 ml.) of the glycerol solution was diluted to 1000 ml. and an aliquot (10 ml.) was treated with 0.02 M sodium metaperiodate (1 ml.). Standard solutions (10 ml.) of formaldehyde (16 µg./ml. and 8 µg./ml.) and a portion (10 ml.) of water were similarly treated with periodate. Samples (1 ml.) were withdrawn after 0.5, 1 and 2 hr. and added to 0.04 M sodium sulphite solution (0.5 ml.) and the colour developed in the usual way with chromotropic acid reagent (10 ml.) followed by 4.6% thiourea (1 ml.). The optical densities (1 cm. cells) showed that oxidation was complete within 0.5 hr. and that the strength of the glycerol solution was 2.70% (i.e. 97% purity).

A solution of glucose (19.2 mg.) in water (100 ml.)

and a solution of cellobiose (27.9 mg.) in water (50 ml.) were prepared. A portion (10 ml.) of each of these was added to deionised water (ca. 200 ml.). 0.027% Glycerol solution (5 ml.) was also added to water (ca. 200 ml.). Each solution was cooled to 2°, and 4 mM sodium metaperiodate (25 ml.) was added and the volume made up to 250 ml. An iodate blank was prepared with 1.88% ethylene glycol (2 ml.), 4 mM sodium metaperiodate (25 ml.) and water to a final volume of 250 ml.

Determination of the reduction of periodate was carried out by withdrawing samples (5 ml.) which were diluted to 50 ml. for optical density measurements. The periodate and iodate solutions gave constant readings of 0.400 and 0.057 respectively. The release of formic acid was estimated using samples (25 ml.) which were added to ca. 1% ethylene glycol solution (2.5 ml.) and kept for ca. 1 hr. at 2° before titration. Samples of the periodate solution were used as blank determinations.

The results for glycerol were:

Time (hr.)	1	2	4
Reduction of periodate (mol.)	1.9	1.9	1.9
Release of formic acid (mol.)	1.07	1.07	0.99

The results for glucose are shown in Fig. 8 and those for cellobiose in Fig. 7.

Release of formic acid from maltose and laminaribiose in dilute solution at 2°.

A solution of maltose (22.1 mg.) in water (50 ml.) and a solution of laminaribiose (23.1 mg.) in water (50 ml.) were prepared. Release of formic acid was estimated as above using portions (10 ml.) (Fig. 7).

Release of formaldehyde from maltose and laminaribiose at 2°.

This was carried out exactly as described for laminarin samples (p. 32). Portions (10 ml.) of maltose solution (16.36 mg.; 50 ml.) and of laminaribiose solution (23.1 mg.; 50 ml.) were used (Fig. 9)

Release of formaldehyde from laminaritriose at room temperature.

This was carried out as above using portions (10 ml.) of a solution of laminaritriose (12.2 mg.) in water (25 ml.) (Fig. 9). The purity of the sample was checked by the phenol/sulphuric acid method on a solution (2.55 mg.; 50 ml.). Glucose content found: 102%.

Reduction of periodate and release of formaldehyde by laminaribiose at room temperature.

A solution of laminaribiose (20.62 mg.) in water

(50 ml.) was prepared. The oxidation was carried out as above but on twice the scale. The reduction of periodate was followed by dilution of samples (0.5 ml.) to 100 ml. An iodate blank containing 0.94% ethylene glycol (20 ml.) and 0.3 M sodium metaperiodate (1 ml.) was also sampled (Fig. 9).

Reduction of periodate and release of formaldehyde by laminaribiitol at 2°.

The above procedure for the oxidation of laminaribiose was adopted. Portion (20 ml.) of a solution of laminaribiitol (29.17 mg.; 50 ml.) were used (Fig. 11).

Release of formaldehyde by laminaribiitol with 57 mM periodate at 2°.

A portion (5 ml.) of the laminaribiitol solution (29.17 mg.; 50 ml.) was treated with water (3.5 ml) and 0.3 M sodium metaperiodate (2 ml.). Blank solutions and a reference standard solution containing formaldehyde were prepared appropriately. Samples (1 ml.) were withdrawn and added to M sodium sulphite (1 ml.), samples (2 ml.) from the blank containing sulphite being used. Ethanol (4 ml.) was added and the estimation continued in the usual way (Fig. 11).

Reduction of periodate and release of formaldehyde by laminaribiitol in dilute solution at room temperature.

A solution of laminaribiitol (8.09 mg.; 50 ml.) was prepared. A portion (20 ml.) was treated with 0.03 M sodium metaperiodate (1 ml.). Appropriate blank solutions were also prepared. Samples (1 ml.) were added to 0.1 M sodium sulphite (0.5 ml.) followed by ethanol (4 ml.). Samples (2 ml.) of the supernatant were treated with chromotropic acid reagent (20 ml.). Thiourea solution (1 ml.) was added and the optical densities were determined using 4 cm. cells (Fig. 12). The reduction of periodate was checked by diluting portions (2 ml.) to 50 ml.

Time (hr.)	2.5	4.5
Reduction of periodate (mol.)	2.0	2.2

Release of formaldehyde from mannitol in acetate buffer (pH 3.5) at room temperature.

Mannitol (19.05 mg.) was dissolved in water (100 ml.). A portion (5 ml.) was treated with 0.2 M acetate buffer (5 ml.), water (2 ml.) and 0.3 M sodium metaperiodate (3 ml.). Samples (0.5 ml.) were added to M sodium aulphite (0.5 ml.) followed by chromotropic acid reagent (9 ml.) and thiourea solution (2 ml.) as usual.

Time	(hr.)	0.5	1	2	24	48
Release of formaldehyde	(mol.)	1.72	1.73	1.80	1.86	1.95

Release of formaldehyde from 1-O-β-laminaribiosyl mannitol at 2°.

Portions (10 ml.) of a solution (15.9 mg.; 25 ml.) were treated as previously described for laminarin samples (p. 32).

Time (hr.)	1	6	24	54
Release of formaldehyde (mol.)	0.95	0.95	0.95	0.95

Release of formic acid from laminaribiosyl mannitol in dilute solution at 2°.

A sample (6.95 mg.) was dissolved in water (ca.200ml.) and the procedure described for glucose and cellobiose was then used.

Time	(hr.)	.33	.67	1.0	1.5	2.0
Release of formic acid	(mol.)	2.75	2.90	2.92	2.96	2.94

SECTION I: THE COMPONENT SUGARS OF LAMINARIN

Mannitol is recognised as a structurally significant component of laminarin, and fucose, which is invariably found in trace quantities in hydrolysates of laminarin, is regarded as arising from contaminating fucoidin. Controversy exists, however, over the presence of mannose. Smith and Unrau[48] and Chesters and Bull[49] have claimed that mannose is a component sugar while no evidence for the presence of mannose was found by Cunningham[115], by Beattie, Hirst and Percival[47] or in the large scale partial acid hydrolysates of Peat *et al.*.[40]

It may be noted that mannose is present in many algae. Among the brown algae, some *Fucus* species[116] contain mannose, while a β-1,4-linked mannan from the red alga *Porphyra umbilicalis*[117] has been characterised. Hydrolysed extracts from several green algae[118,119,120] also contain mannose and a further β-1,4-linked mannan has been obtained from *Codium fragile*.[121,122]

The formation of mannose as an artefact during the hydrolysis of a polysaccharide, or during the neutralisation of the hydrolysate, requires consideration before significance can be attached to its presence. Epimerisation of glucose during neutralisation of sulphuric acid hydrolysates with barium carbonate[59] and

basic ion-exchange resins[123] has been reported, but only fructose was identified. Duff[124] has shown that alkaline material present in chromatographic paper may also cause epimerisation. The formation of mannose, however, was conclusively demonstrated by Sowden[125] and this was subsequently confirmed by other workers.[126,127] It is reported[58] that Biodeminrolit resin saturated with carbon dioxide to convert the strongly basic component to its carbonate form does not cause epimerisation. An investigation[128] into the reported presence of mannose in cellulose indicated that the mannose was an artefact produced by Amberlite IR 45 OH^- resin or by glucose oxidase which was used to remove the large excess of glucose from hydrolysates.

The action of acid on glucose is known to produce, in addition to products of reversion synthesis, 1,6-anhydroglucopyranose and-furanose[129], 5-hydroxymethyl furfural and laevulinic acid.[130] Recently, acid epimerisation of glucose to give fructose was reported[131] but the presence of mannose was not established.

In addition, therefore, to the examination of several samples of laminarin, the procedures adopted by Smith et al.[48] and Chesters et al.[49] have also been studied.

Examination of Laminarin samples.

The separation of small amounts of mannose from glucose may be achieved electrophoretically,[132] by ion-exchange chromatography,[80,133] or by paper chromatography. In the present work, the most useful paper chromatographic solvent was the boric acid solvent, 9/1/1, which gave R_G values of 1.5, 3.0 and 3.3 for mannose, fucose and mannitol respectively.

A synthetic mixture of glucose(82%), mannitol (15%) and mannose (3%), which approximated to the analysis given by Smith for his sample, was chromatographed and the presence of mannose was readily revealed by the aniline phthalate reagent. It was further found that mannose was detectable in a mixture of glucose (99%) and mannose (1%). Hydrolysates of laminarin samples were neutralised with barium carbonate and examined under similar conditions. Sample B.B.2 revealed a trace of fucose but no mannose. Somewhat larger amounts of fucose were found in the Borax sample, Smith's sample, S.L.5. and samples 5 and 6 but the presence of mannose could not be established in any hydrolysate.

The sensitivity of the detection of mannose was greatly increased by preliminary fractionation of the neutralised hydrolysate on a column of Dowex 50W X 8 resin in the Ba^{++} form. A synthetic mixture of

glucose (89%) and mannose (11%) was applied to the column and an almost complete separation was observed. A hydrolysate of the Borax sample was neutralised with barium carbonate, deionised with Biodeminrolit CO_3^{--} and fractionated on the column. A small amount of mannose was detected, closely followed in the eluate by mannitol and a small amount of fructose, the identity of which was confirmed with the resorcinol reagent. In view of the presence of fructose, the experiment was repeated without deionisation with Biodeminrolit CO_3^{--}. Fructose was no longer detectable but a trace of mannose was still present. This procedure was then repeated using sample B.B.2., sample S.L.5. and Smith's sample, with the result that trace quantities of mannose were found in all except B.B.2.

A quantitative experiment was carried out using the Borax sample. A hydrolysate was fractionated and the fractions containing mannose were combined. The reducing power found by cuprimetric titration indicated a hexose content of 5.4 mg. while paper chromatography showed the presence of glucose and fucose, each present to a greater extent than mannose. The remaining fractions of the hydrolysate were combined and found to contain 915 mg. of glucose. The presence of mannose to the extent of about one part in 500 is therefore indicated.

It is noticeable that laminarin samples which contain larger proportions of fucose show detectable traces of mannose, while the extensively purified sample B.B.2. contains little fucose and no mannose. Samples of soluble laminarin are more difficult to purify than those of insoluble laminarin, spontaneous deposition from aqueous solution being available for the latter only. It seems likely, therefore, that the very small amounts of mannose found could arise from contaminating material and are not structurally significant.

The results of an examination of the crude laminarin extracted from L. saccharina support this view. A hydrolysate contained, in addition to glucose and mannitol, a substantial amount of fucose, a little xylose and a trace of mannose. After purification to give sample 6, only fucose was detectable by paper chromatography.

Investigation of the procedures of Smith et al. and Chesters et al.

Smith and Unrau[134] hydrolysed laminarin by refluxing with N hydrochloric acid for 12 hr. The hydrolysate was evaporated to dryness, without neutralisation, and the residue was refluxed for 20 hr. with methanolic 2% hydrogen chloride to convert the sugars into their

glycosides; methyl α-mannoside (R_G 2.7) and methyl α-glucoside (R_G 1.9) being readily separable using solvent 5/2/7. Methyl α-mannoside was detected chromatographically in the products from several samples of laminarin and characterised by mixed melting point.

Chesters and Bull treated an enzymic hydrolysate of the Borax sample with methanolic hydrogen chloride and detected methyl α-mannoside chromatographically using the same solvent.

A mixture of glucose (97.5%) and mannose (2.5%) was, therefore, methanolysed and examined by paper chromatography. Methyl α-mannoside could be detected using silver nitrate reagent. A simulated hydrolysis with dilute sulphuric acid, followed by neutralisation and evaporation to dryness, before methanolysis did not interfere with this result.

A portion of the Borax sample was hydrolysed (1.5 N sulphuric acid; 3 hr. at 100°) and the hydrolysate was neutralised, evaporated to dryness and methanolysed. Methyl α-mannoside could not be detected in the products. Methanolysis, under more drastic conditions, of the unhydrolysed polysaccharide gave the same result. It is therefore concluded that the laminarinase preparations of Chesters and Bull were responsible for the formation of mannose in their work.

The procedure of Unrau was applied to laminarin B.B.2. A small proportion of a substance indistinguishable from methyl α-mannoside in solvents 6/4/3 and 4/1/5 was obtained. Chromatography in 5/2/7, however, showed instead two components both of which had R_G values greater than methyl α-mannoside. These results were also observed when "Analar" glucose was treated in the same manner with aqueous, followed by methanolic, hydrogen chloride.

The artefact was separated from the products of the reaction with laminarin B.B.2. by thick paper chromatography using solvent 6/4/3. Rechromatography in 5/2/7 resolved the substance into the two components of higher chromatographic mobility, It is clear that the substance is a mixture of at least two components, which does not include methyl α-mannoside.

Hydrolysis of the substance replaced the two non-reducing components by four reducing components, of which three were tentatively identified.

Component	Relative amount
Glucose	++
Xylose	+
Arabinose	tr
Unidentified (R_G 1.49 in 6/4/3)	tr

Xylose and arabinose, however, were present in a hydrolysate of an aqueous extract of 3MM paper,(v.i.) and although the glucose found may have arisen in part from a slight contamination of the material with methyl α- glucoside, it is probable that the material reverts to glucose on hydrolysis.

Notwithstanding failure to find methyl α-mannoside, the action of aqueous hydrochloric acid alone on glucose was investigated, because complete conversion to glycosides was not achieved in the previous experiments and the methods for their detection on paper are less sensitive than those for free sugars. Chromatography of the products in 4/1/5 showed the presence of two components with similar mobilities to 1,6-anhydroglucose. Separation of these was carried out using 3MM paper and the same solvent. The leading edge of the area containing glucose was also excised with a view to searching for mannose.

The two products could be distinguished, by their mobilities in 10/4/3 and 4/1/5 and by their reaction with aniline phthalate, from 1,6-anhydroglucose, 3,6-anhydroglucose and 5-hydroxymethyl furfural. The mobilities of both components resembled those of the 6-deoxy sugars, fucose and rhamnose, and one gave a very similar colour with aniline phthalate, but neither of

them have been identified. Examination of the leading edge of the glucose region in solvents 9/1/1 and 10/4/3 indicated the presence of a trace of material corresponding to mannose.

The action of aqueous hydrochloric acid on glucose has been repeated by Mr.J.C.Patterson who obtained substantially the same results. It is evident that the formation of mannose as an artefact to an extent approaching 2.4% or ca. 12%[48] is unlikely. No explanation of Unrau's results can be advanced on this basis.

It is therefore concluded that none of the laminarin samples examined contained more than ca. 0.2% of mannose and that this minute amount cannot be structurally significant.

Water-soluble carbohydrates from Whatman No. 3MM paper.

Huffman et al.[135] obtained a polysaccharide by repeated extraction of Whatman No. 1 paper with water. This was shown to contain xylose (54%), arabinose (16%), a uronic acid (14%) and glucose (11%) as main components.

In order to check the source of some of the products obtained (v.s.) after the elution of compounds from chromatograms, lengths of Whatman No, 3MM paper were extracted with water. Chromatography of the concentrated

extract revealed the presence of a non-reducing compound with R_G 0.56 in solvent 4/1/5 (cf. sucrose, 0.61; xylobiose, 0.57; maltose, 0.42; cellobiose, 0.38; lactose, 0.28; and raffinose, 0.155).

Chromatography of a hydrolysate of the extract showed the presence of xylose, a smaller amount of glucose and traces of arabinose and galactose. This result, similar to that obtained by Huffman et al., indicated that small amounts of xylose and arabinose observed previously probably arose from this source.

Experimental

Fractionation of sugars on Dowex 50WX 8 Ba^{++} resin.

Resin (140 g.) was regenerated as previously described (p.28). After being packed and back-washed, the column settled to 70 X 1.8 cm.

A mixture of glucose (200 mg.) and mannose (25 mg.) in water (5 ml.) was placed on the column and elution was carried out with water. The flow rate of one fraction (2.5 ml.) per 25 mn. was achieved using a capillary tube which carried the eluate above the level of the resin inside the column. This conferred the advantage that the column could not run dry. Paper chromatographic examination (solvent 9/1/1) showed that

glucose was the main component in fractions 30-41 while mannose was the main component in fractions 42-55. The maximum concentrations of the two components occurred around fractions 36 and 46 and overlapping of the components occurred only to a slight extent. With use the column settled further, which resulted in the elution of compounds occurring some 6 fractions earlier.

The elution of laminarin hydrolysates could be followed by examination of fractions on paper, without chromatographic development, using the silver nitrate reagent. The maximum concentration of glucose, around fraction 30, was followed by a distinct peak around fraction 43 arising from mannitol. The latter provided a useful reference marker for location of mannose, present around fraction 39. Further detail was obtained by chromatography in 9/1/1 with silver nitrate or a periodate reagent - the latter being required to locate mannitol in the presence of borate.

Borax laminarin (1 g.) was hydrolysed (1.5 N sulphuric acid, 100 ml.; 3 hr. at 100°). The neutralised hydrolysate was stirred with Biodeminrolit resin which had previously been treated with carbon dioxide. The mixture was filtered and the filtrate concentrated to 5 ml. and applied to the Dowex resin column. Fructose was detected in the mannitol-

containing fractions. Further samples (1 g.; except for Smith's sample, 0.15 g.) were examined similarly except that treatment with Biodeminrolit resin was omitted.

Borax laminarin (1 g.) was hydrolysed and fractionated as above. Examination of the fractions showed that mannose should be contained largely in fractions 37 - 41. These were combined and the volume was made up to 50 ml. The remaining fractions (25-36, 42-50) were combined and the volume made up to 1,000 ml. Portions (3 ml. and 1 ml. respectively) were diluted to 5 ml. for estimation by cuprimetric titration.

Hydrolysis and methanolysis of laminarin B.B.2. and glucose.

Samples (ca. 150 mg.) of laminarin B.B.2. and glucose were refluxed with aqueous N hydrochloric acid (4 ml.) for 12 hr. Each solution was cooled and evaporated to a syrup. Each residue was co-distilled with methanol (3 ml.) and dried *in vacuo* over calcium chloride for one day.

Methanolic 2% hydrogen chloride (4 ml.) was added and each solution was refluxed for 20 hr., neutralised with silver carbonate, concentrated and examined by paper chromatography.

Action of aqueous hydrochloric acid on glucose.

Glucose (0.5 g.) was refluxed with N hydrochloric acid (13.2 ml.) for 12.5 hr. The solution was evaporated to a syrup which was dissolved in water, centrifuged to remove insoluble material, and re-evaporated. Chromatography in 4/1/5 gave two components with R_G values of 2.0 and 2.3 (cf. 1,6-anhydroglucose, 2.3, and 5-hydroxymethyl furfural, 5).

Components of Whatman No. 3MM paper.

A strip (35 X 15 cm.) of paper was pulped with water (200 ml.). The residue was removed by filtration and re-extracted with water (100 ml.). The combined extracts were evaporated, centrifuged to remove residual fibre and examined by paper chromatography.

The remainder of the extract was hydrolysed with 1.5 N sulphuric acid (25 ml.). Identification of the products was based on chromatography in 6/4/3 and 18/3/1/4, the latter solvent distinguishing between mannose (R_G 1.3) and arabinose (R_G 1.6).

SECTION II: THE POSITION OF MANNITOL IN LAMINARIN

The presence of mannitol in laminarin was first observed by Peat, Whelan and Lawley[31]. A partial acid hydrolysate afforded[40] significant quantities of mannitol, 1-O-β-glucosyl mannitol and 1-O-β-laminaribiosyl mannitol, together with trace amounts of 1,6-di-O-β-glucosyl mannitol and 1-O-β-isomaltosyl mannitol. It was suggested that acid reversion might account for the presence of the last two components. It is evident that 1- substituted mannitol residues must be incorporated in laminarin but it cannot be concluded that this is the sole mode of linkage. The rate of acid hydrolysis of different types of linkage varies considerably[136] and the effect of this during partial acid hydrolysis cannot be fully predicted. e.g.[137] In view of the symmetry of mannitol, a proportion of 1,6-disubstituted mannitol residues in laminarin could not, on the above evidence, be excluded.

Goldstein, Smith and Unrau[45] applied the sequence of reactions of periodate oxidation, reduction with borohydride and acid hydrolysis to laminarin (see p. 18; Fig. 5a, 5b). Ethylene glycol was not detected among the products. As this finding was inconsistent with the presence of 1- substituted mannitol residues in

laminarin, disubstitution was suggested. Of the two possibilities, 1,2- and 1,3- disubstitution, the latter was inconsistent with the absence of erythritol in the products; 1,2- disubstitution was therefore advanced. Unrau[134] has further claimed the isolation of 3,4,5,6-tetramethyl mannitol from a hydrolysate of methylated laminarin. This compound was characterised as a di-p-nitrobenzoate with m.p. 80° and $[\alpha]_D$ + 12°.

The problem was therefore re-examined by procedures involving periodate oxidation and methylation.

The mannitol content of laminarin.

It is necessary for valid interpretation of the results of periodate oxidation to obtain an independent estimate of the mannitol content of laminarin. Peat et al.[40] devised a method involving hydrolysis of laminarin, followed by fermentation with yeast to remove glucose and a subsequent determination of optical rotation in the presence of molybdate. Two samples of laminarin were used and results of 1.7% and 2.6% were obtained. In the present work, chromatographic separation on thick paper of the hydrolysis products was employed.

A preliminary experiment with a synthetic mixture of mannitol and glucose in the molar ratio of 1 to 40 was

carried out. The large excess of glucose prevented adequate chromatographic separation using 18/3/1/4; use was therefore made of the boric acid solvent, 9/1/1, and of the periodate/permanganate reagent for the detection of mannitol. Glucose and mannitol were estimated, after elution, by the phenol/sulphuric acid method, and by measurement of the release of formaldehyde on periodate oxidation in bicarbonate buffer respectively.

Two possible sources of interference were apparent; borate may interfere[132] with the phenol/sulphuric acid method, and fucose, present as a minor impurity in laminarin hydrolysates, was not readily separable from mannitol using 9/1/1. Oxidation in bicarbonate buffer of fucose might be expected to produce acetaldehyde instead of formaldehyde but the conditions under which this interferes with the determination of the latter are not clear.[87] The estimation of glucose was therefore repeated after treatment of a portion with methanolic hydrogen chloride. An unchanged result indicated that no interference by borate had occurred. Similarly, a control oxidation to which fucose was added showed that this would not interfere with the estimation of mannitol. The estimated molar ratio of mannitol to glucose was 1 to 38.

Laminarin B.B.2. and sample 6 were hydrolysed and

the neutralised hydrolysates chromatographed as above. Mannitol contents of 2.1% and 2.5% respectively were observed.

Periodate oxidation of laminarin.

The immediate release of formaldehyde whem laminarin is oxidised at 2° was determined for several samples.

Sample	B.B.2.	6	Borax	Smith
Formaldehyde (mol./ anhydrohexose unit)	0.024	0.027	0.029	0.034

It has been shown (p. 47) that 1-O-β-laminaribiosyl mannitol yields one mol. of formaldehyde under these conditions. Mannitol contents of 2.4% and 2.7% are therefore indicated by the results for sample B.B.2. and sample 6. These are in reasonable agreement with the values obtained above and are therefore incompatible with 1,6- disubstitution of mannitol residues.

On oxidation in dilute solution at 2°, 1-O-β-laminaribiosyl mannitol was found (p. 47) to liberate three mol. of formic acid. 1- substituted mannitol residues in laminarin should, therefore, also give three mol. of formic acid, while 1,2- disubstitution, as postulated by Goldstein et al., would lead to the formation of only two mol. of formic acid per mannitol residue. Laminarin was therefore oxidised under these

conditions and the release of formic acid, which became constant after 0.5 to 1 hr., was determined. Using the mannitol contents determined above by the release of formaldehyde, the results were;

Sample	B.B.2.	Borax	Smith
Formic acid (mol./mannitol residue)	3.0	3.1	3.1

This lends strong support to 1- substitution rather than 1,2- disubstitution of the mannitol residues.

Laminarin Polyalcohol.

In view of the results obtained by periodate oxidation it was necessary to re-examine laminarin polyalcohol for the presence of ethylene glycol as a constituent.

Moore et al.[138] have indicated that losses caused by volatility occur during paper chromatography of ethylene glycol but the technique was nevertheless useful for qualitative detection. Preliminary work showed that glycol could just be detected in a mixture of glucose, glycerol and glycol in the proportions 95.5: 3.1: 1.4 (W/W). Glycolaldehyde, which is present in hydrolysates of laminarin polyalcohol, masked the region of the chromatogram appropriate to glycol but it could be completely removed, together with glucose, by

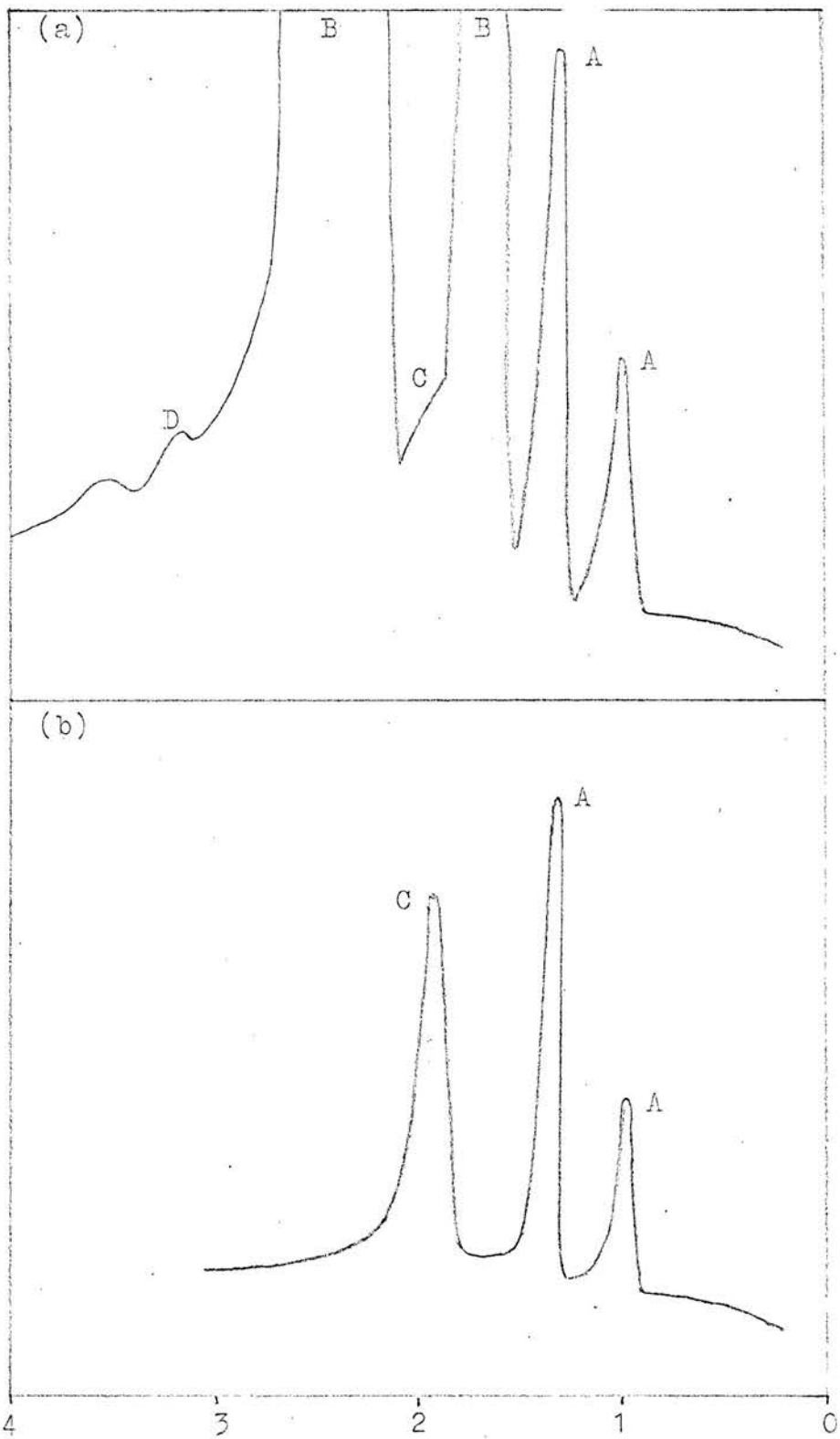
Amberlite IRA 400 OH^- resin. Control experiments showed that no artefacts were produced and that the recovery of glycol was quantitative when a column of resin was washed with 2.6 bed volumes of water.

Losses of ethylene glycol can occur during concentration of aqueous solutions (cf. Neish,[139] for concentration of solutions of glycerol). When a solution (14.25 mg; 1 l.) was evaporated to dryness, using a rotary film evaporator with a bath temperature below 40°, only 2.60 mg. of ethylene glycol remained. Collection of the glycol in the distillate was found to be impracticable and the procedure adopted was to avoid evaporation to complete dryness. Quantitative recoveries were obtained in this way.

Laminarin polyalcohol was prepared from laminarin B.B.2., hydrolysed and the neutralised hydrolysate passed through a column of Amberlite IRA 400 OH^- resin. The eluate (3 bed vol.) was concentrated and found, by paper chromatography, to contain arabitol, glycerol and ethylene glycol. The mixture was separated by cellulose column chromatography, enabling estimation and characterisation of the components to be carried out. Determination of the release of formaldehyde on periodate oxidation was used for estimation of glycerol and glycol. Under the conditions used, the presence of a proportion

of butanol and ethanol in the solvent did not noticeably retard the periodate oxidation (cf. Taylor *et al.*;[101] Honeyman *et al.*[140]) but caused a reduction of 5 - 10% in the final optical density. Comparison was therefore made with standard solutions containing the appropriate proportions of butanol and ethanol.

A sample (7.22 g.) of laminarin polyalcohol gave arabitol (48 mg.), glycerol (227 mg.) and glycol (38 mg.). The yield of arabitol expected on the basis of a D.P. of 24 and 57% of M chains (see Section IV) is about 124 mg. While some formyl ester hydrolysis and consequent further oxidation will have occurred during the oxidation period (7 days at 2°) of the laminarin, an apparent loss of ca. 60% is excessive. It has since been found that higher polyhydric alcohols are retained by strongly basic ion-exchange resin (see Appendix B) and that ca. 60% of the arabitol would remain on the resin column after the use of three bed volumes of eluant. The yield of glycerol indicated 1.3 "triol" groups per molecule of laminarin and suggested the presence of a small degree of branching. A 2.4% content of 1- substituted mannitol residues would give rise to ca. 54 mg. of ethylene glycol. It is considered that the observed recovery of 38 mg. is in satisfactory agreement with the proposed 1- substitution of mannitol



A.
2,3,4,6-Me_4 Glucose.

B.
2,4,6-Me_3 Glucose.

C.
2,3,4,5,6-Me_5 Mannitol.

D.
2,4-Me_2 Glucose.

Fig. 14. GAS-LIQUID CHROMATOGRAM OF THE METHANOLYSIS PRODUCTS FROM

(a) METHYLATED LAMINARIN.

(b) METHYLATED 1-O-β-GLUCOSYL MANNITOL.

in view of inevitable manipulative losses, particularly on the cellulose column which incompletely resolved the components.

Methylation studies.

Methylation of laminarin should therefore result in the formation of 2,3,4,5,6-penta methyl mannitol residues. The high molecular weight fraction of methylated laminarin, prepared by Dr.A.G.Ross,[42] was methanolysed and the products were examined by gas-liquid chromatography. A small shoulder was observed on a peak arising from the main component, 2,4,6-trimethyl glucose (Fig.14a). The relative retention time of this shoulder was 3.66 on the butan-diol succinate polyester column and 1.97 on the polyphenyl ether column.

The identity of a sample of 1-O-β-glucosyl mannitol, kindly provided by Dr.D.H.Hutson, was confirmed by periodate oxidation. Measurement of the release of formaldehyde (by Mr.J.R.Stark) and formic acid gave values of 0.96 mol. and 3.7 mol. (theoretical: 1 mol.; 4 mol., respectively)

Methylation[141] of the sample with dimethyl sulphate, in dimethyl formamide and dimethyl sulphoxide, gave a product which was methanolysed and examined by gas-liquid chromatography. (Fig. 14b). Apart from the

methyl 2,3,4,6-tetramethyl glucosides, only one other component was observed, the latter possessing relative retention times of 3.67 and 1.94 on the columns used above. The excellent agreement indicates that 2,3,4,5,6-pentamethyl mannitol is a constituent of methylated laminarin and thus confirms the 1- substitution of the mannitol residues. Estimation and characterisation of pentamethyl mannitol from methylated laminarin is still required but all the present evidence is strongly in favour of 1- substituted mannitol.

Some of the results described in this section have been published and a reprint of the communication is included at the end of the thesis.

Experimental

Analysis of glucose/mannitol mixtures.

Glucose (652.4 mg.) and mannitol (16.3 mg.) were dissolved in water (7 ml.). The solution was streaked on Whatman No. 3MM paper until sufficient material was present for subsequent analysis; applications of the same solution were used to provide reference markers. After chromatographic separation in 9/1/1, three equal areas of paper were cut out corresponding to glucose, mannitol and paper blank. Each piece was soaked in

water (20 ml.) and the eluate was separated by filtration through glass wool.

Portions of the paper blank and glucose eluates were diluted five-fold and the recovery of glucose was estimated to be 6.6 mg. by the phenol/sulphuric acid method. Further portions (1 ml.) of the diluted solutions were evaporated to dryness and the residues treated with methanolic 1% hydrogen chloride (10 ml.). After re-evaporation to dryness, the glucose contents were again estimated giving the same result as before.

Portions (7 ml.) of the paper blank and mannitol eluates were evaporated to dryness. Water (3 ml.), 0.5 M sodium bicarbonate (0.5 ml.) and 0.02 M sodium metaperiodate (1 ml.) were added. Samples (1 ml.) were withdrawn after 2.5 and 16.5 hr., added to 0.2 molar sodium sulphite (0.5 ml.) and the estimation of formaldehyde was completed in the usual way. The results, which were constant, indicated a recovery of 0.176 mg. of mannitol, leading to a molar ratio of glucose to mannitol of 38 to one. Two standard solutions of ethylene glycol were also oxidised, to one of which an equimolecular amount of fucose had been added. The addition of the latter proved to have no effect on the estimation of formaldehyde.

Laminarin samples B.B.2. and 6 (ca. 50 mg. each)

were hydrolysed and the neutralised hydrolysates chromatographed as above. The glucose eluates, diluted sevenfold for estimation, indicated recoveries of 10.8 mg. and 10.6 mg. respectively. Portions (3 ml.) of the mannitol eluates were treated with bicarbonate and periodate as above. Paper blank determinations were carried out simultaneously, this being particularly necessary for accurate estimation of mannitol where blank values were appreciable e.g. typical optical densities (O.D.) observed were:

	Paper blank	Sample B.B.2.	Sample 6.
O.D.	0.077	0.312	0.351

Mannitol recoveries of 0.234 mg. and 0.273 mg. were indicated, giving 2.1% and 2.5% for the mannitol contents of the laminarin samples.

Periodate oxidation of laminarin at 2°.

a) Release of formaldehyde.

Portions (10 ml.) of solutions of sample B.B.2. (114.2 mg.; 25 ml.), sample 6 (91.7 mg.; 25 ml.), Borax sample (92.0 mg.; 25 ml.) and Smith's sample (26.5 mg.; 25 ml.) were treated as previously described (p. 32). The release of formaldehyde (mol./anhydrohexose unit) was:

Time (hr.)	B.B.2.	6*	Borax	Smith
1	0.024	0.027	0.028	0.034
6	0.023	0.026	0.030	0.034
24	0.025	-	0.031	0.039

* sampled at 1.5 and 5.5 hr.

b) Release of formic acid in dilute solution.

Solutions (ca. 200 ml.) of sample B.B.2. (79.1 mg.), Borax sample (88.0 mg.) and Smith's sample (51.8 mg.) were oxidised by the procedure described previously (p. 50). The release of formic acid (mol./mannitol residue) was:

Time (hr.)	B.B.2.	Borax	Smith
0.25	2.73	-	2.84
0.5	2.94	3.09	-
0.75	3.00	3.03	3.09
1.0	2.96	3.05	3.09
1.5	2.94	2.94	3.14
2.0	2.92	3.24	3.12
3.0	2.98	2.97	-

Paper chromatography of polyhydric alcohols.

The boric acid solvent, 9/1/1, proved to be unsatisfactory for ethylene glycol and glycerol as the use of either silver nitrate or periodate/permanganate reagents produced a discoloured background which had a rear boundary at R_F 0.3. Preliminary washing of the

paper with solvent failed to give satisfactory results. The most useful solvent was n-butanol/ethanol/water (4/1/5), which gave R_G values of 3.7, 2.7, 1.9 and 1.4 for glycol, glycerol, erythritol and arabitol respectively. Ammoniacal silver nitrate was used for their detection.

Removal of reducing compounds by strongly basic ion-exchange resin.

Throughout this work, Amberlite IRA 400 OH^- analytical grade resin was used. Columns were regenerated[142] with N sodium hydroxide (6 bed vol.) and washed slowly with deionised water until the effluent was neutral to phenolphthalein. Compounds were eluted with deionised water.

Glycolaldehyde solution (20 mg.; 2ml.) was applied to a column (10.5 x 1.7 cm.) which was eluted at a rate of 2 ml. per min. Seven fractions (10 ml.) were collected, concentrated and examined by paper chromatography in 4/1/5. No material was detected apart from traces of ionic material of low R_F value. Glucose (150 mg.; 4 ml.) was applied to a fresh column and none was detected in the eluate (8 x 10 ml. fractions). A mixture of ethylene glycol (6.2 mg.) and glycolaldehyde (18 mg.) in water (2 ml.) was similarly treated and glycol alone was readily detected in the eluate, being

present in fractions 2-6. A mixture of glucose (90 mg.), glycerol (4.0 mg.) and glycolaldehyde (2.2 mg.) was heated at 100° for 2 hr. with 1.5 N sulphuric acid (20 ml.). The solution was cooled, neutralised and evaporated to 3 ml. After passage through the resin column no material other than glycerol was obtained; this was present in fractions 2-6.

Recovery of ethylene glycol from strongly basic resin column.

A 1.94% solution of ethylene glycol was prepared by weighing and standardised by estimation of the release of formaldehyde on periodate oxidation. An aliquot (1 ml.) was diluted to 1,000 ml. and portions (10 ml.) were treated as described (p. 49) for glycerol. An oxidation period of 0.5 hr. was found to be adequate and was used for all subsequent estimations. The observed concentration was 1.88% (i.e. 97% purity).

A portion (3 ml.) of the glycol solution, diluted to 200 ml., was applied to a column (40 x 6.6 cm.) and eluted at a rate of 100 ml. per min. The eluate (3.5 l.; 2.6 bed vol.) was diluted to 4 l. and portions (10 ml.) oxidised as above. A recovery of 54 mg. was observed (96%).

Evaporation of aqueous ethylene glycol solutions.

The above standard solution of ethylene glycol was employed and analyses were performed by estimation of formaldehyde as described above.

a) A solution (14.25 mg.; 1 l.) was evaporated to dryness with a bath temperature of 35 -40°. The residue was redissolved in water and re-evaporated. The final residue contained 2.6 mg. of ethylene glycol (18%).

b) A solution (18.8 mg.; 1 l.) was treated similarly except that a bath temperature of 60° was used. Analysis showed that the residue and the distillate contained 2.2 mg. and 7.5 mg., respectively, of ethylene glycol.

c) The evaporation was repeated using a solution (18.8 mg.; 1 l.) and a bath temperature of 80° but was discontinued before the residue had been evaporated to dryness. The residue (17 ml.) was found to contain 17.9 mg. of glycol (95%).

d) It was finally shown that a solution (18.8 mg.; 10 ml.) could be evaporated carefully, using a bath temperature of 25°, to 46.6 mg. containing 18.1 mg. of glycol (96%).

Preparation of laminarin polyalcohol.

Laminarin B.B.2. (8.97 g.) was dissolved in water (400 ml.) and the solution cooled to 2°. Sodium metaperiodate solution (5 g.; 100 ml.) was added and the

oxidation was allowed to proceed for 7 days at 2°. Mechanical shaking was used as the laminarin precipitated after oxidation for 4 hr. The oxopolysaccharide was recovered[143] by the addition of 6 N hydrochloric acid(44ml.), with rapid stirring, followed by sodium iodide solution (27 g.; 20 ml.). The mixture was stirred into cold ethanol (6 l.) and the oxopolysaccharide collected by decantation and centrifugation, washed four times with methylated spirits and dried in vacuo over calcium chloride. Yield: 8.85 g.

The product was dissolved in warm water (350 ml.) and cooled to room temperature. Potassium borohydride solution (1.5 g.; 15 ml.) was added and, after 26 hr., a further addition (0.75 g.; 7.5 ml.) was made. After 42 hr. the solution was neutralised with glacial acetic acid and stirred into ethanol (4.3 l.). The precipitate was collected, washed and dried as before. Yield: 8.58 g. A portion, when dried under reduced pressure at 100° over phosphorus pentoxide, showed a loss in weight of 6.3%. The overall yield of laminarin polyalcohol was therefore ca. 90%.

Hydrolysis of laminarin polyalcohol.

Laminarin polyalcohol (100 mg.) was hydrolysed with 1.5 N sulphuric acid (25 ml.) for 3 hr. at 100°. The

neutralised hydrolysate was concentrated to 3 ml. and applied to a column of Amberlite IRA 400 OH^- resin. The eluate was concentrated, chromatographed (4/1/5; ammoniacal silver nitrate) and found to contain arabitol, glycerol and ethylene glycol.

Cellulose column chromatography.

A column (53 x 3 cm.) of Whatman coarse cellulose powder was packed dry and washed with water (2 l.) followed by n-butanol/ethanol/water (4/1/5) for several days.

The column was tested with a mixture of ethylene glycol (80 mg.), glycerol (270 mg.) and erythritol (200 mg.) dissolved in a mixture (3 ml.) of butanol, ethanol and water. The flow rate was one fraction (5 ml.) per 4 min. Fractions 63-70 contained ethylene glycol, fractions 71-78 contained glycerol and fractions 80-98 contained erythritol, although slight tailing and resultant overlapping of components was also observed.

Estimation of glycol and glycerol in the presence of butanol and ethanol.

A solution of ethylene glycol (1.88%; 3 ml.) was treated with 4/1/5 (40 ml.), ethanol (3 ml.) and the volume made up to 100 ml. with aqueous ethanol

(1:1 v/v), thereby avoiding the formation of two liquid phases. A solution of glycerol (2.70%; 10 ml.) was treated with 4/1/5 (70 ml.), ethanol (10 ml.) and the volume again made up to 100 ml. with aqueous ethanol (1:1 v/v). Portions (1 ml.) of these solutions were diluted with water to 50 ml. and 100 ml., respectively, and portions (10 ml.) of these diluted solutions oxidised as previously described (p. 49). The oxidation appeared to be complete after 0.5 hr., but it was necessary to add 5.5% and 8.2% respectively to the observed optical densities to obtain values equal to those obtained by oxidation in aqueous solution.

Large-scale hydrolysis of laminarin polyalcohol.

Laminarin polyalcohol (7.22 g.) was hydrolysed with 1.5 N sulphuric acid (500 ml.) for 3 hr. at 100°. The neutralised hydrolysate was concentrated to 200 ml. and passed through a column (40 x 6.6 cm.) of Amberlite IRA 400 OH^- resin. The eluate (4 l.) was evaporated to 1.66 g. and transferred to the cellulose column (53 x 3 cm.). Fractions (2.5 ml.) were collected and examined by paper chromatography. Fractions 132-149, which contained ethylene glycol, were combined and the volume was made up to 100 ml. with aqueous ethanol (1:1 v/v). A portion (1 ml.) was diluted to 100 ml.

and portions (10 ml.) of the diluted solution were oxidised as before. A correction of 5.5% was added, since the proportions of butanol and ethanol were the same as in the preliminary experiment, giving an observed yield of 38 mg. of ethylene glycol. A blank determination using fractions collected before the elution of glycol produced no detectable formaldehyde.

Fractions 150-184, which contained glycerol, were combined and the volume was made up to 250 ml. The estimation was performed in a similar manner to the above. After adding a correction of 5.0%, which was indicated by a control experiment with the appropriate amounts of butanol and ethanol, a yield of 227 mg. was determined.

A minute trace of a compound corresponding in chromatographic mobility to erythritol was found in fractions 185-188. Fractions 189-250, which contained arabitol, were evaporated to dryness. Yield: 48 mg.

Characterisation of ethylene glycol.

The solution of ethylene glycol was evaporated to 75 mg. The residue was heated at 100° with pyridine (3 ml.) and p-nitrobenzoyl chloride (0.5 g.) for 15 min. and the solution poured into water (15 ml.). The product was extracted with benzene (15 ml.) and the

benzene extract was washed successively with dilute hydrochloric acid, dilute sodium carbonate solution and water and dried over potassium carbonate. The benzene was removed and the residue treated with charcoal and recrystallised from ethanol. The ethylene glycol di-p-nitrobenzoate had m.p. and mixed m.p. 144-145°.

Characterisation of glycerol.

The solution of glycerol was evaporated to a syrup which was treated, as above, with pyridine (5 ml.) and p-nitrobenzoyl chloride (2 g.). The solution was poured into sodium bicarbonate solution (50 ml.) and extracted with chloroform (30 ml.). The product was purified in the usual way and finally recrystallised twice, with some difficulty, from mixtures of chloroform and petroleum ether; m.p. 188-192°. An authentic specimen of glycerol tri-p-nitrobenzoate had m.p. 194-196° and gave a mixed m.p. 193-196°.

Characterisation of arabitol.

The arabitol was heated at 100° with pyridine (3 ml.) and benzoyl chloride (0.5 ml.) for 15 min. The solution was poured into dilute sodium carbonate solution (10 ml.) and the product was extracted with chloroform (10 ml.). After purification and

recrystallisation from ethanol, the arabitol pentabenzoate had m.p. and mixed m.p. 150-152°.

Periodate oxidation of 1-O-β-glucosyl mannitol.

a) Release of formaldehyde. (by Mr.J.R.Stark.)

A portion (1 ml.) of a solution of glucosyl mannitol (6.02 mg./ml., determined by the anthrone method[144]) was diluted to 10 ml. A portion (2 ml.) of the diluted solution was treated with 4 mM periodate (5 ml.) and water (3 ml.) at 2°, and samples (1 ml.) were analysed at intervals. A constant release of 0.96 mol. of formaldehyde was observed.

b) Release of formic acid.

A portion (20 ml.) of a solution of glucosyl mannitol (0.568 mg./ml., determined by the phenol/sulphuric acid method) was treated at 2° with water (ca. 100 ml.), 0.1875 M periodate (100 ml.) and the volume made up to 250 ml. Samples (25 ml.) were removed, treated with ethylene glycol (2 ml.), and kept for 0.5 hr. before titration with 0.0095 N barium hydroxide in the usual way.

Time (hr.)	0.5	5	16.5	25	41
Formic acid (mol.)	3.10	3.65	3.76	3.76	3.80

Methylation of 1-O-β-glucosyl mannitol.

Glucosyl mannitol (50 mg.) was dissolved in N,N-dimethyl formamide (2.5 ml.) and dimethyl sulphoxide (2.5 ml.). The solution was cooled to 0° and barium oxide (1 g.) and barium hydroxide (0.5 g.) were added. Dimethyl sulphate (1.5 ml.) was added dropwise over 1 hr. with continuous stirring. Stirring was maintained for 28 hr., the temperature having been allowed to rise to room temperature after 8 hr.

Ammonia solution (1.5 ml.; 0.880) was added and stirring continued for 1 hr. The product was extracted with chloroform (20 ml.), the oily residue being re-extracted with further portions of chloroform (2 x 5 ml.). The combined extracts were washed with water until neutral, dried over sodium sulphate and evaporated to dryness under reduced pressure. Residual solvent was removed by the application of a high vacuum at room temperature for 1 hr., by which time a reasonably constant weight had been reached. Yield: 33 mg. (48%).

A portion was methanolysed (p. 24) and examined by gas-liquid chromatography.

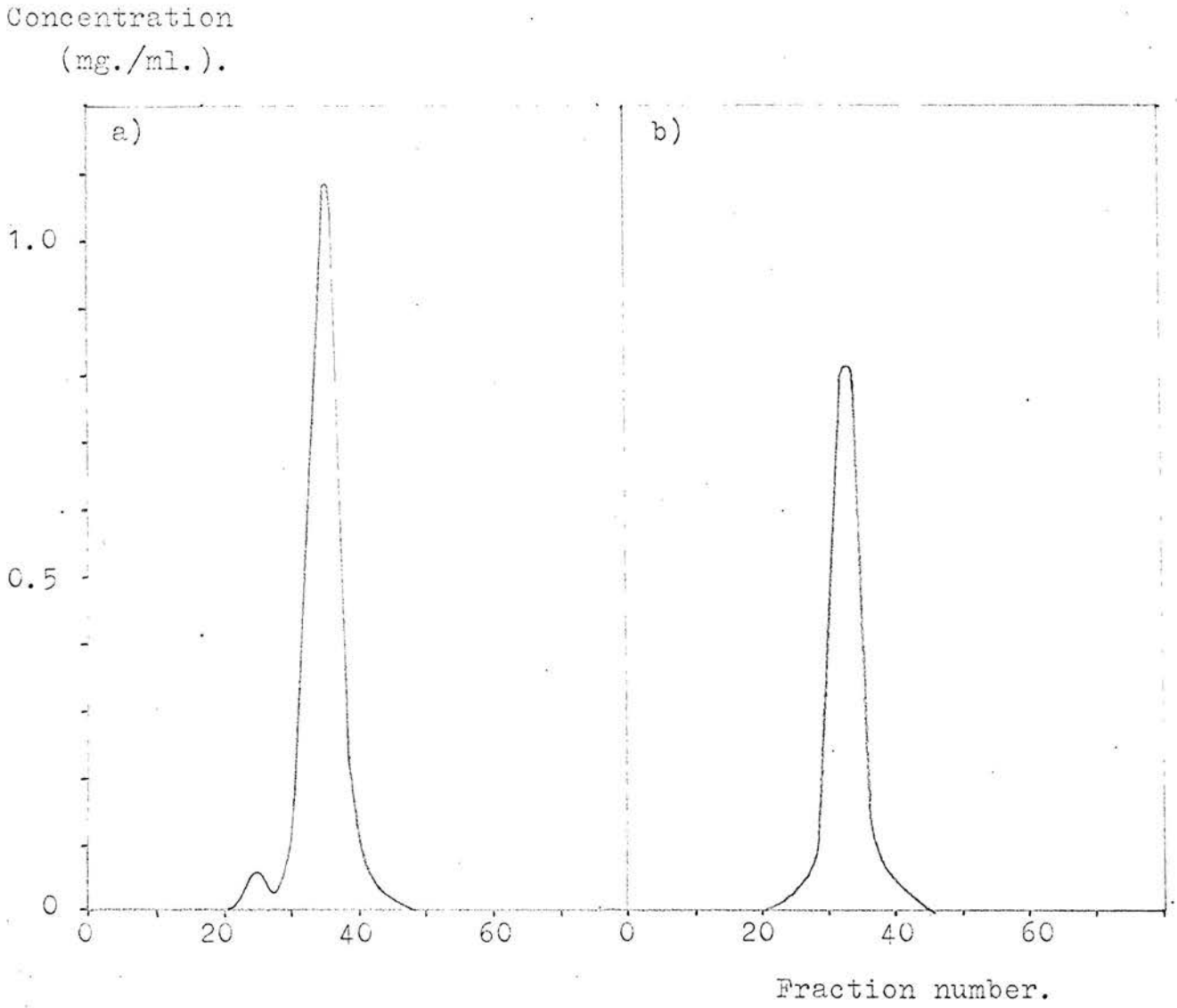
SECTION III: THE SITE OF 1,6-LINKAGES IN LAMINARIN

The presence of 1,6- linkages in laminarin was first established[40] by the isolation of oligosaccharides containing 1,6- linkages from a partial acid hydrolysate. The disaccharide fraction of a partial hydrolysate of insoluble laminarin contained gentiobiose and laminaribiose in the molar ratio of one to 70, and, in view of the possible contribution by reversion synthesis[129] to the proportion of the former, and the lower rate of hydrolysis[136] of 1,6- linkages, it is likely that the proportion of these in the polysaccharide may be less than this. No evidence for branching, in the form of 3,6-di-O-β-glucosyl glucose was obtained, but appreciable amounts of "branched" trisaccharides have not been found in partial acid hydrolysates of glucans which are known to be branched. A possible exception to this is the recent isolation[145], in 0.9% yield, of a sugar tentatively identified as 4,6-di-O-α-glucosyl glucose from the α-glucan, pullulan, but in general, the absence of a branched trisaccharide cannot be construed as strong evidence against branching. Evidence that 1,6-inter-residue, as opposed to inter-chain, linkages do not occur was provided by Hirst *et al.*[43] who demonstrated that repeated degradation of laminarin by Barry's method

failed to produce readily dialysable fragments.

A sample of methylated laminarin (D.P. 58), prepared by Dr.A.G.Ross from laminarin B.B.2., contained[42] an amount of tetramethyl glucose equivalent to a C.L. of 23, no 2,3,4-trimethyl glucose, and 7.2% of 2,4-dimethyl glucose. While the last sugar may have arisen partly by demethylation, the presence of branching is established by the difference between the D.P. and the C.L. and it is apparent that the 1,6- linkages are present entirely as inter-chain, rather than inter-residue, linkages. Unrau[134] has, however, claimed the isolation of 2,3,4-trimethyl glucose from methylated laminarin and Beattie, Hirst and Percival[47] subsequently obtained evidence for the presence of the same sugar.

Further evidence in support of 1,6- inter-residue linkages was the isolation[46] of ethylene glycol after treating either the G-chain or M-chain fraction of laminarin to periodate oxidation, borohydride reduction followed by a mild acid hydrolysis to cleave acetal but not glucosidic linkages, and a second oxidation, reduction and complete acid hydrolysis. (p.18, Fig.6b). It has been shown in Section II, however, that ethylene glycol would arise from 1- substituted mannitol residues, thereby invalidating the above inference with respect to the M-chain fraction.



a) Fractionation before hydrolysis.

b) Fractionation after hydrolysis.

Fig. 15. GEL-FILTRATION OF LAMINARIN POLYALCOHOL BEFORE AND AFTER MILD ACID HYDROLYSIS.

Smith and Unrau also adduced as evidence for 1,6-inter-residue linkages the presence of combined glycerol after the M-chain fraction had been treated to periodate oxidation, borohydride reduction and mild acid hydrolysis (p.19, Fig. 6b). Present work on lichenin polyalcohol has suggested that the conditions of mild acid hydrolysis (0.1 N sulphuric acid; 10 hr. at room temperature) given by Unrau[60] do not completely hydrolyse acetal linkages. It is possible therefore that the glycerol was not combined glucosidically but may have been present in acetal linkage such as would arise from non-reducing glucose terminal residues.

This section describes experiments designed to detect the fragmentation of laminarin by the Smith degradation procedure, and a re-examination of methylated laminarin.

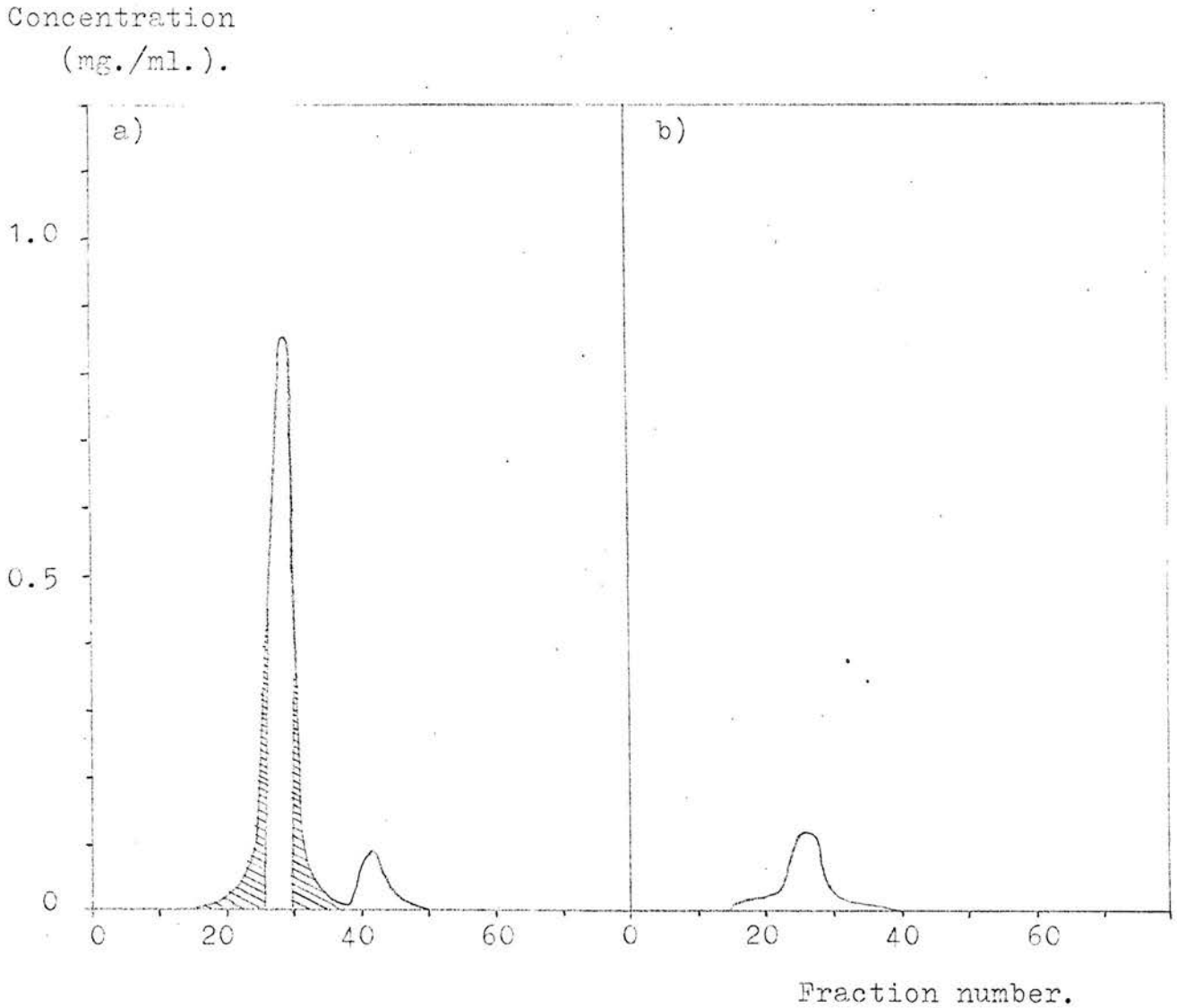
Examination of laminarin polyalcohol.

a) Paper Chromatography.

The sample of laminarin polyalcohol was shown to be oligosaccharide-free. After a mild acid hydrolysis (0.1N sulphuric acid; 18 hr. at room temperature) oligosaccharides could not be detected.

b) Gel-filtration.

A column of Sephadex G-50, which should fractionate



a) Fractionation together with reference pentasaccharide.

b) Refractionation of the tails of the peak in a).

Fig. 16. GEL-FILTRATION OF LAMINARIN POLYALCOHOL AFTER MILD ACID HYDROLYSIS.

in the range of D.P. 6 to 60, was used in this work. The elution of material was followed using the phenol/ sulphuric acid method, and the total recovery, determined in two cases, was quantitative within experimental error.

Laminarin polyalcohol was eluted as a sharp peak which was preceeded by a minor (3%) peak apparently consisting of a high molecular weight fraction (Fig.15a). The latter, of which only 1 mg. was obtained, was not further investigated. After mild acid hydrolysis, laminarin polyalcohol gave only one sharp peak (Fig.15b). Apart from the disappearance of the minor component, no difference in heterogeneity could be discerned. Owing to the settling of the column with use, the maximum concentration in the eluate occurred two fractions earlier, viz. in fraction 33 compared with 35. No overall decrease in molecular weight is therefore apparent. Glycerol was, however, located in fractions 50-54, demonstrating that cleavage of acetal links had occurred during the mild hydrolysis.

1-O-β-Laminaritetraosyl mannitol was added as a marker to a portion of a mild acid hydrolysate before gel-filtration, and the absence of low molecular weight material in the latter was clearly demonstrated by their complete resolution (Fig.16a). The residual material present in the leading and tailing edges of the

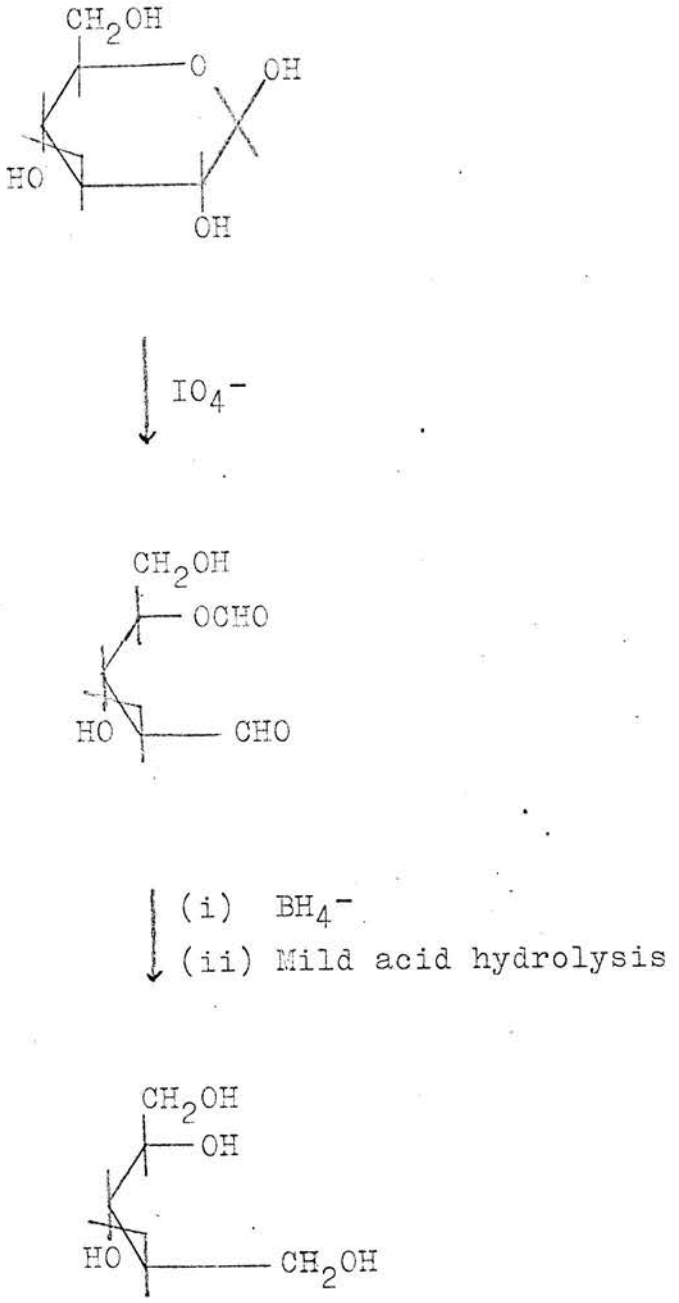
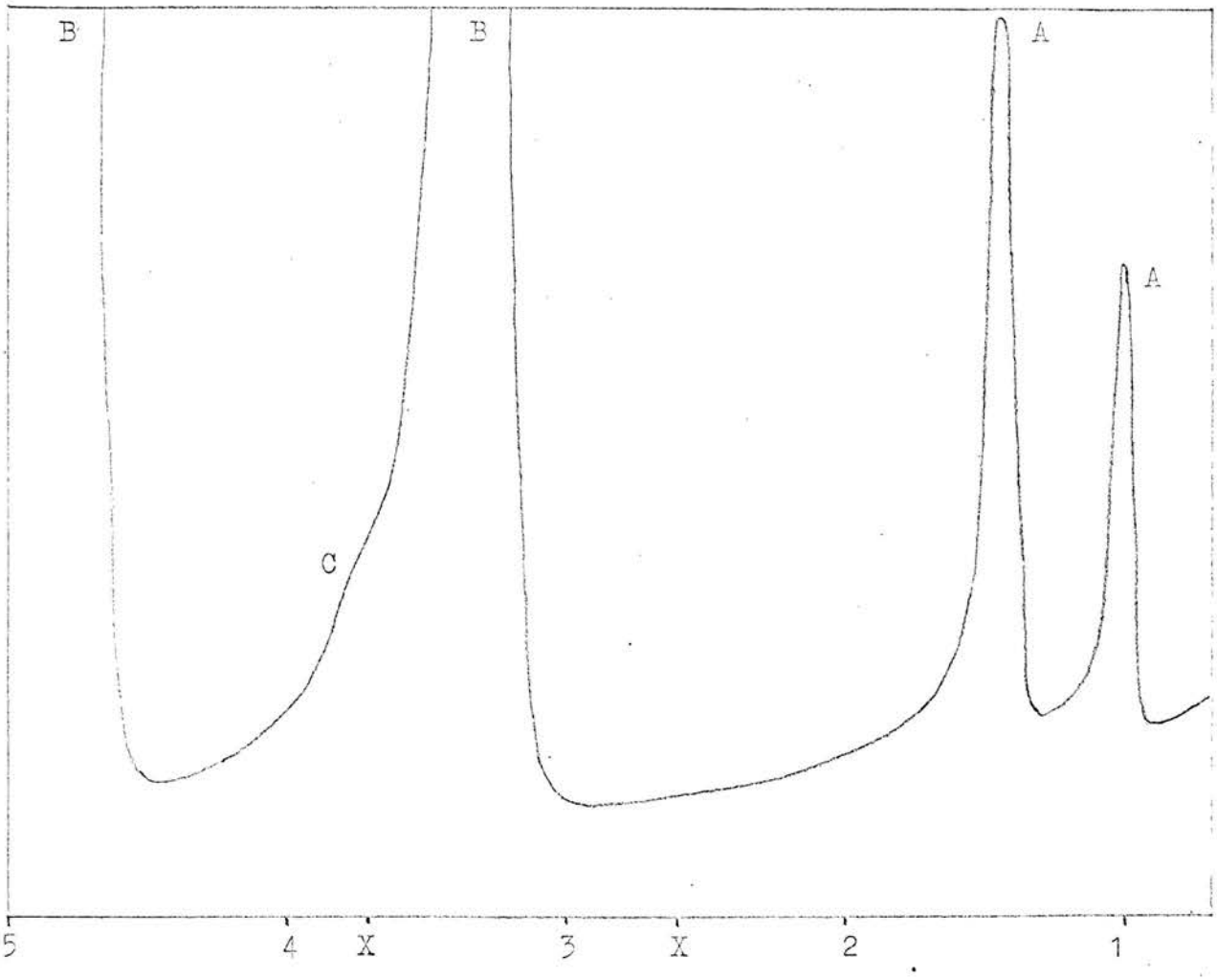


Fig. 17. SMITH DEGRADATION OF THE REDUCING GLUCOSE TERMINAL RESIDUE.

polysaccharide peak, which together corresponded to about 50% of the original recovery, were combined and re-fractionated. The unimodal peak observed (Fig. 16b) indicated that, while some fractionation may occur, the proportion of material of D.P. substantially different from the mean value was rather small.

The sequence of reactions, oxidation, reduction and mild acid hydrolysis, converts 1- substituted mannitol residues into ethylene glycol residues (Fig. 5a), reducing glucose terminal residues into 2- substituted D-arabitol residues (Fig. 17), and cleaves 1,6- inter-residue linkages to produce 3- substituted D-glycerol residues (Fig. 6b). Periodate oxidation of a mild acid hydrolysate should therefore liberate an amount of formaldehyde corresponding to one mol. for each original reducing glucose residue and an additional one mol. for each original 1,6- inter-residue linkage. The yield arising from the former (see Section IV) should be 0.018 mol. per anhydrohexose residue.

A mild acid hydrolysate was therefore fractionated by gel-filtration to remove glycerol, glycolaldehyde and sodium sulphate. On periodate oxidation, the polymeric material liberated 0.019 mol. of formaldehyde/ anhydrohexose unit indicating that 1,6- inter-residue linkages cannot occur to any great extent. The amount



Relative retention time (approx.) on butan-diol succinate polyester column.

A. 2,3,4,6-Me_4 Glucose.

B. 2,4,6-Me_3 Glucose.

C. 2,3,4,5,6-Me_5 Mannitol.

X. 2,3,4-Me_3 Glucose.

Fig. 18. GAS-LIQUID CHROMATOGRAM OF THE METHANOLYSIS PRODUCTS FROM METHYLATED LAMINARIN.

of glycerol released was also estimated, by periodate oxidation, giving an apparent C.L. of 40. This latter result is clearly inadmissible as the release of formic acid on oxidation of laminarin B.B.2. indicates one "triol" group per 19 anhydrohexose units. Studies on lichenin polyalcohol have indicated that complete hydrolysis of acetal linkages requires stronger conditions than those used here. The quantitative results from the above Smith degradation cannot therefore be regarded as reliable.

Examination of methylated laminarin.

The sample of methylated laminarin prepared by Dr. A.G.Ross was re-examined. Absence of 2,3,4-trimethyl glucose was confirmed by paper chromatography, and by gas-liquid chromatography (Fig. 18).

The procedure used by Unrau for the hydrolysis of methylated laminarin involved refluxing with methanolic 1.5% hydrogen chloride for 18 hr. followed by evaporation and treatment of the residue with boiling N hydrochloric acid for 24 hr. The hydrolysate was then neutralised and evaporated. Demethylation may occur[57] during both of these treatments, probably most extensively during the latter. Pure tetramethyl glucose was therefore subjected to the treatment with N hydrochloric acid and

the products were examined by paper chromatography. A small proportion of mixed trimethyl glucoses was observed together with a trace of dimethyl glucose. While the significance of Unrau's finding is perhaps equivocal, the procedure of Beattie, Hirst and Percival appears to be above suspicion and no explanation of their results can be offered.

It would be interesting to examine further samples of laminarin, but it seems that laminarin B.B.2. does not contain 1,6- inter-residue linkages.

Experimental

Examination of laminarin polyalcohol.

The sample prepared (p. 82) from laminarin B.B.2. was used.

a) Paper chromatography.

Solvent 10/4/3 which can readily resolve laminaridextrins up to a D.P. of 7 (R_G 0.13), and silver nitrate reagent were used. Laminarin polyalcohol (12 mg.) was dissolved in water (4 ml.). The solution was cooled to room temperature and 0.3 N sulphuric acid (2 ml.) added. After 18 hr. the solution was neutralised with barium carbonate and examined in the usual way. Oligosaccharide material could not be detected.

b) Gel-filtration.

A column (120 x 1.7 cm.) of Sephadex G-50 (medium grade) was prepared and washed with water for 18 hr. before use. Concentrations of samples were determined by the phenol/sulphuric acid method and a volume of 10 ml. was usually applied to the column. Fractions (5.1 ml.) were collected and portions (1 ml.) suitably diluted for analysis by the phenol/sulphuric acid method, the total recovery being estimated from the area enclosed by the graph. With continued use the flow rate of the column gradually decreased from 1 ml./min. to 0.33 ml./min.

i) Laminarin polyalcohol (29 mg.) was fractionated with a recovery of 109%.

ii) Laminarin polyalcohol (ca. 50 mg.) was dissolved in water(14 ml.). The solution was cooled to room temperature and 0.3 N sulphuric acid (6 ml.) added. After 20 hr. the solution was neutralised to pH 4 with 0.5 N sodium hydroxide and a sample (18 mg.; 10 ml.) applied to the column. (Recovery 115%). The glucose content fell to zero at fraction 45; fractions 45 - 70 were combined in batches of five and chromatographed (6/4/3; silver nitrate). Glycerol was detected in fractions 50 - 54.

iii) Laminarin polyalcohol was hydrolysed as in ii).

Laminaritetraosyl mannitol (ca. 2 mg.) was added to a portion (ca. 20 mg.; 10 ml.) of the neutralised hydrolysate and the mixture fractionated as before.

iv) The portions remaining, after analysis, of fractions 15 to 26 and 30 to 37 from the eluate of iii) were evaporated to a sample (5 ml.) and refractionated. The omitted fractions, 27, 28 and 29, were estimated to contain 10.5 mg. i.e. about half of the original amount used.

v) A portion of mild acid hydrolysate (21.3 mg.) was fractionated and the eluted polysaccharide located, with negligible consumption of material, by analysing fractions immediately flanking the expected position of the polysaccharide peak. Fractions 18 - 40 were combined, concentrated and freeze-dried. A portion (18.4 mg.) of the product was dissolved in water (10 ml.), cooled to 2° and treated with 0.3 M sodium metaperiodate (0.5 ml.). Samples were withdrawn at intervals for estimation of formaldehyde in the usual way.

Fractions 44 to 64 were combined for the estimation of glycerol. The remaining solution in the intervening fractions, 41 to 43, was also collected in order to confirm that these fractions contained a negligible amount of glycerol. Both solutions were evaporated to 5 ml. and each was passed successively through a column

(16 ml.) of Amberlite IR 120 H^+ resin and a column (30 ml.) of Amberlite IRA 400 OH^- resin to remove glycolaldehyde and sodium sulphate. Each column was washed with water (4 bed vol.) after the application of each solution and the final eluates, combined with the appropriate washings, were evaporated to a few ml. The volumes of the two solutions were adjusted to 50 ml. and 10 ml. respectively. Paper chromatography (4/1/5) showed the presence of glycerol only. Portions (5 ml.) were treated with 0.02 M sodium metaperiodate (0.5 ml.) and samples (1 ml.) were withdrawn at intervals and added to 0.04 M sodium sulphite (0.5 ml.) for estimation of formaldehyde. Recoveries of glycerol were 295 µg. and 5 µg. confirming that the glycerol was present almost entirely in fractions 44 to 64. An apparent chain length of 40 is indicated by the release of 0.30 mg. of glycerol from 21.3 mg. of polysaccharide.

Examination of methylated laminarin.

a) Paper chromatography (200/17/1; aniline phthalate) of a hydrolysate of methylated laminarin showed that 2,3,4-trimethyl glucose (R_{Me_4G} 0.72) was absent (cf. R_{Me_4G} 0.59 for 2,4,6- trimethyl glucose).

b) Gas-liquid chromatography (butan-diol succinate polyester) of a methanolysate of methylated laminarin

confirmed the absence of 2,3,4-trimethyl glucose (Fig.18).

Action of aqueous hydrochloric acid on tetramethyl glucose.

Chromatographically pure tetramethyl glucose (8 mg.) was heated at 100° in a sealed tube with N hydrochloric acid (4 ml.) for 26 hr. The solution was cooled, neutralised with silver carbonate and examined by paper chromatography (200/17/1; aniline phthalate). Small amounts of trimethyl glucoses and a trace of dimethyl glucose were observed.

SECTION IV: HETEROGENEITY OF LAMINARIN

Characteristics of laminarin samples include the mannitol content, the proportion of reducing glucose residues which, with the above, indicates the average D.P. of the sample, and the degree of branching of the molecules. These features take different values for different samples of laminarin and may provide an explanation of their different behaviour with respect to solubility. Little is yet known about heterogeneity within, as opposed to between, samples, such as the distribution of branching points and molecular weights, and their association with each other and with the termination of molecules by mannitol residues.

This section outlines the main characteristics of several laminarin samples, and describes the results of experiments designed to investigate the heterogeneity of one of them.

The mannitol content of laminarin samples.

It has been shown that the initial release of formaldehyde on periodate oxidation at 2° gives an accurate estimate of the mannitol content. Results obtained from four samples have been mentioned in Section II; these will be given here together with the

results from two further samples for comparison.

Sample	B.B.2.	S.L.5.	Borax	Smith	5	6
Mannitol content (%)	2.4	2.9	2.9	3.4	3.0	2.7

The mannitol contents of samples from different sources all lie within the range 2.9 ± 0.5 %.

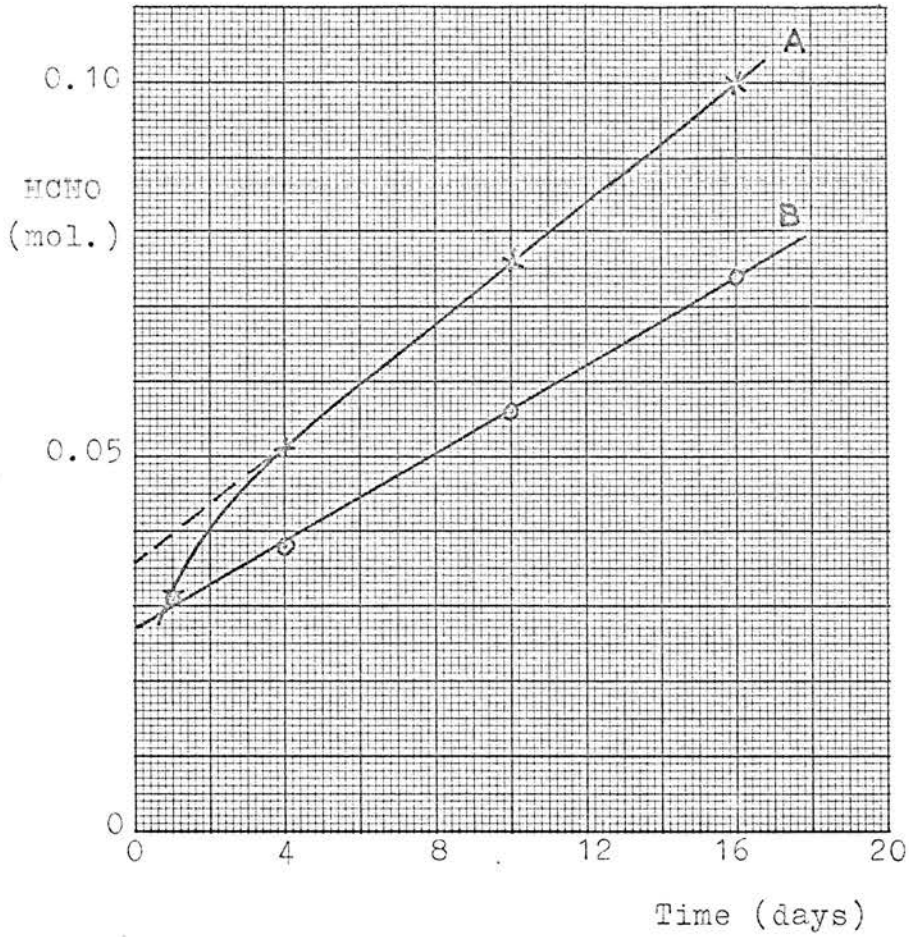
The proportion of reducing glucose terminal residues in laminarin samples.

Four samples of laminarin were oxidised at room temperature (18 ± 2°). The release of formaldehyde (mol./anhydrohexose residue) was:

Time (hr.)	B.B.2.*	S.L.5.	Borax	Smith
4.5	-	0.027	0.031	0.034
31	0.029	0.032	0.039	0.039
78	0.037	0.034	0.043	0.045

* sampled at 24 and 53 hr.

Anderson et al.[42] and Beattie et al.[47] considered that the rate of liberation of formaldehyde from reducing glucose residues was negligible at 2°, but that at room temperature one mol. of formaldehyde would be quantitatively liberated after ca. 2 days. Work on model compounds (p. 42) has substantiated the former proposition, but has indicated that liberation of formaldehyde from laminarin at room temperature should



27.3 mM Periodate at 20°.

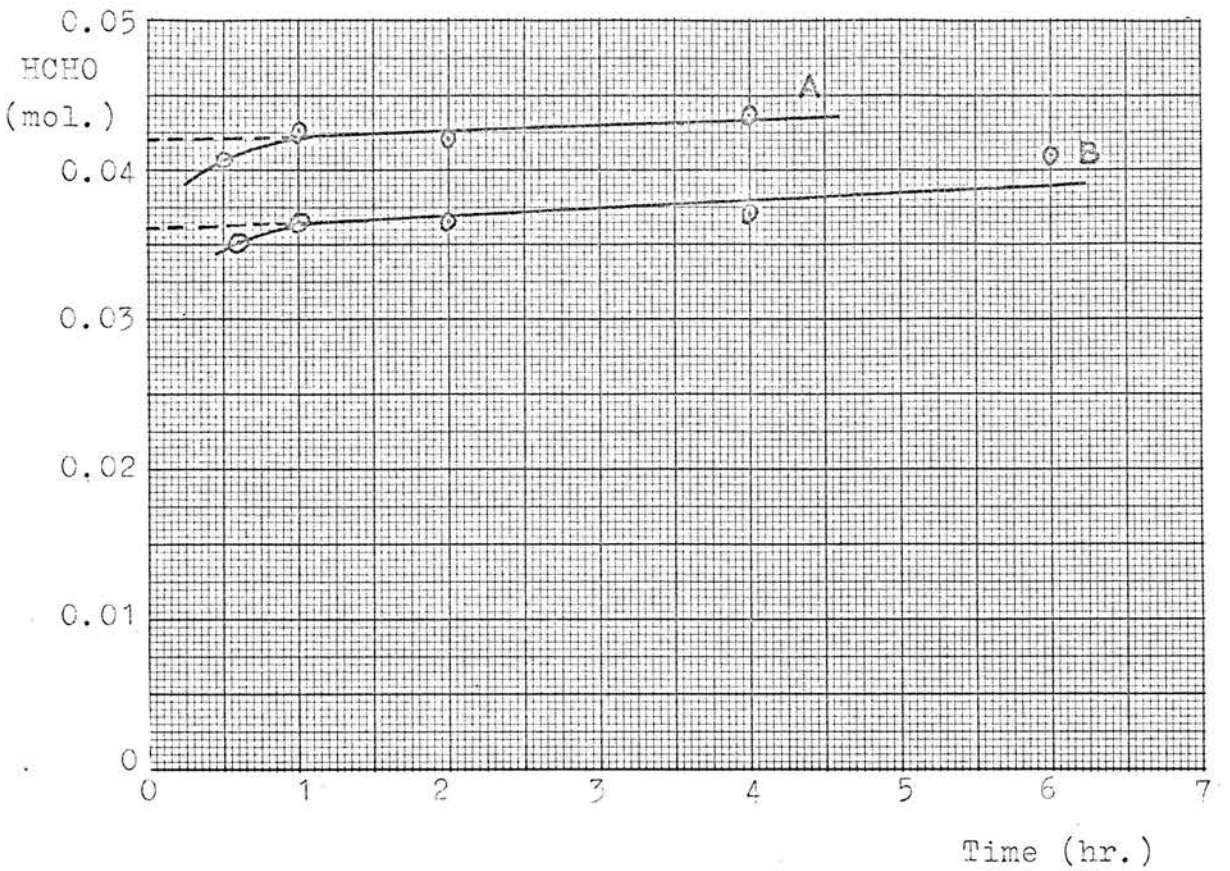
A. Release of formaldehyde from 0.301% sample B.B.2.

B. Release of formaldehyde from 0.325% sample 6.

Fig. 19. PERIODATE OXIDATION OF LAMINARIN.

proceed steadily with continuous overoxidation and that no stage should be discernable when one mol. of formaldehyde per reducing glucose residue was produced.

The oxidation of laminarin at room temperature was therefore studied in more detail using samples B.B.2. and 6 which were oxidised at 20 ± 1° for 16 days (Fig. 19). The release of formaldehyde from sample 6 rose linearly from an initial value of 0.027 mol./anhydrohexose residue which corresponded with the mannitol content. The rate of release of formaldehyde from sample B.B.2. was initially much greater but decreased until after 5 days when it became constant at a value slightly greater than that for sample 6. The linear portion of the latter graph, when extrapolated to zero time, gave an apparent initial release of 0.036 mol./anhydrohexose residue which contrasted with the figure of 0.024 appropriate to the mannitol content. It was observed, however, that while sample 6 had remained in solution throughout the oxidation, sample B.B.2. had precipitated extensively during the initial 5 days. The subsequent rate of oxidation is that of a heterogeneous reaction and the figure of 0.036 is therefore not structurally significant. It may be deduced nevertheless that sample B.B.2. has a higher content of reducing glucose residues than sample 6 from the greater rate of



1.43 mM Periodate at 18°.

A. Release of formaldehyde from 0.073% sample B.B.2.

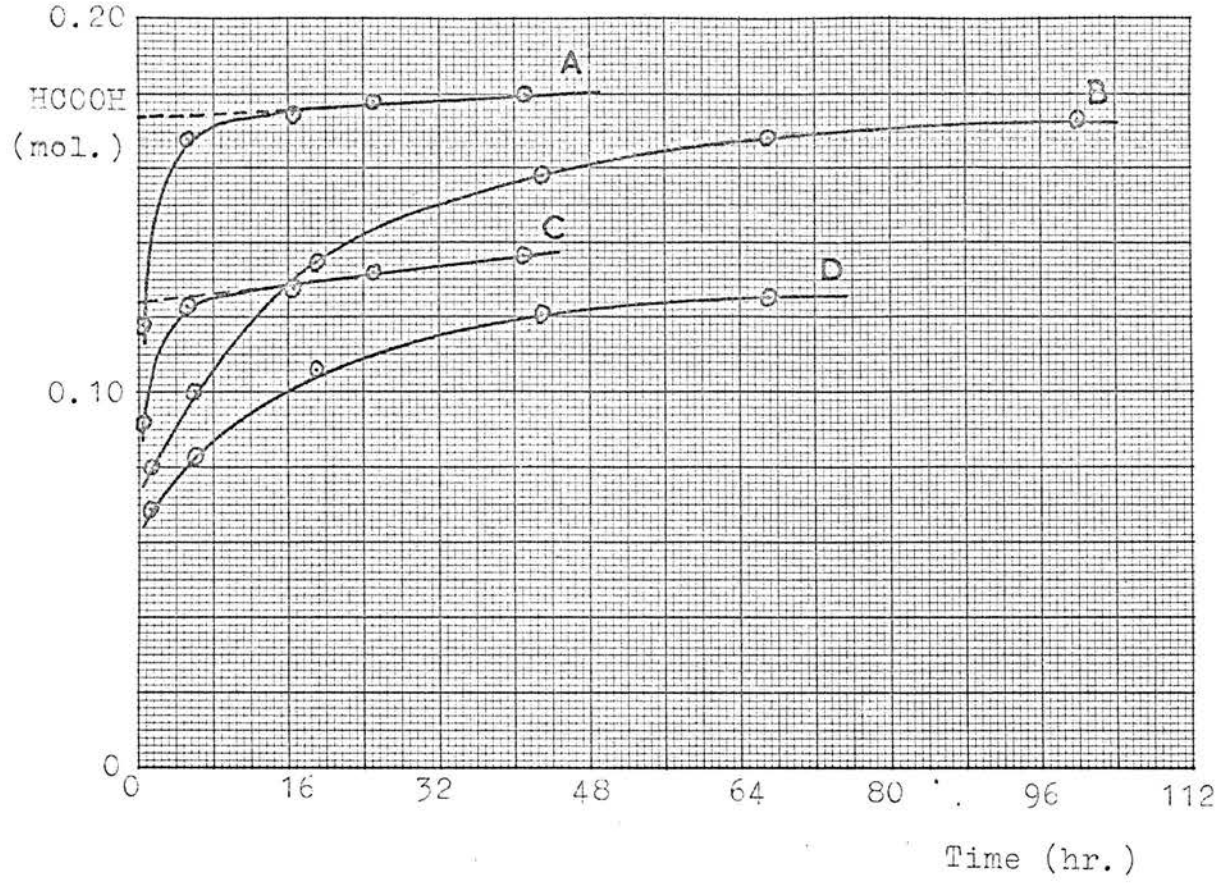
B. Release of formaldehyde from 0.082% sample 6.

Fig. 20. PERIODATE OXIDATION OF LAMINARITOL.

liberation of formaldehyde from the former.

An alternative approach was indicated by work with model compounds. Reduction of laminarin with borohydride to give laminaritol followed by oxidation of the latter with dilute periodate at room temperature should liberate one mol. of formaldehyde from each sorbitol residue in addition to that from each mannitol residue. Laminaritol was therefore prepared from sample 6 and oxidised, together with laminaritol prepared by Dr. F.B.Anderson from laminarin B.B.2. The initial release of formaldehyde was 0.042 and 0.036 mol./ anhydrohexose residue for sample B.B.2. and sample 6 respectively (Fig. 20). Values for the D.P. of 24 and 28 respectively are therefore indicated, and the calculated proportions of G-chains are 43% and 25%. In agreement with the deduction above, the contents of reducing glucose residues are 0.018 and 0.009 mol./ anhydrohexose residue.

The initial release of formaldehyde from laminaritol apparently corresponds roughly with the release of formaldehyde from the appropriate laminarin after oxidation for about 70 hr. under the conditions used. It is therefore possible to suggest from the results of oxidation at room temperature that sample S.L.5. has a similar D.P. to sample 6 but possibly an even lower



Release of formic acid at 2° from:

A. 0.166% Sample 6 with 75 mM periodate.

B. 0.179% Sample 6 with 7.5mM periodate.

C. 0.163% Sample B.B.2. with 75 mM periodate.

D. 0.160% Sample B.B.2. with 7.5mM periodate.

Fig. 21. PERIODATE OXIDATION OF LAMINARIN.

G - chain content, and that the Borax and Smith samples have D.P.s similar to that of B.B.2. Smith's sample has a G - chain content similar to that of sample 6, while the G - chain content of the Borax sample is probably intermediate between that of B.B.2 and sample 6.

The degree of branching of laminarin samples.

Samples B.B.2. and 6 were oxidised at 2° with a limited excess of periodate. Under these conditions it was possible to measure both the release of formic acid and the reduction of periodate. The former, after subtraction of the formic acid obtained from mannitol residues, should give the C.L., while the value of the latter should corroborate the structure thus derived.

The results were, however, unsatisfactory in that the rate of the reaction never became constant, thus preventing the determination of the initial extent of the reaction by extrapolating to zero time (Fig. 21). The results for sample B.B.2. would, in any case, be vitiated by precipitation which occurred after oxidation for 2 days. It may be noted that the release of formic acid observed after oxidation for one hr. was 0.069 and 0.080 mol./anhydrohexose unit for samples B.B.2. and 6 respectively, which corresponds well with the release expected from the oxidation of mannitol residues alone,

viz. 0.072 and 0.081.

It is apparent that the use of a limited excess of periodate merely retards the required oxidation and allows interfering side reactions to gain prominence (cf. Manners and Wright[146]). Repetition of the oxidation with a tenfold increase in the periodate concentration proved successful, giving values of 0.124 (cf.[42] 0.10) and 0.174 mol. of formic acid/anhydrohexose residue for samples B.B.2. and 6 respectively (Fig. 21). Apparent chain lengths of 18.5 and 10.6 are indicated after subtraction of figures of 0.070 and 0.080 to allow for the release of formic acid from mannitol residues. Using the previously determined values of 24 and 28 for the D.P. of the samples, the proportions of "triol" groups per molecule are 1.3 and 2.6. The former value is in excellent agreement with the figure of 1.3 derived from the quantity of glycerol produced on hydrolysis of the polyalcohol from laminarin B.B.2. (p. 74). The average number of branch points per molecule is therefore 0.3 and 1.6, showing that sample 6 has a much higher degree of branching than sample B.B.2. This is the only marked difference between the two samples and may be associated with the occurrence of spontaneous precipitation which is shown by sample B.B.2. only.

Molecular-weight distribution within laminarin samples.

a) Paper chromatography.

Laminarin B.B.2., sample 5 and sample 6 were examined by paper chromatography. No mobile material was detected except in the case of B.B.2. which contained minute traces of reducing sugars with R_G 0.53, 0.39, 0.27, 0.19 and 0.13 in 10/4/3, suggesting the presence of laminaridextrins down to laminaritriose. A plot of log R_G against D.P. was accurately linear[65]. It was necessary to use heat to maintain a sufficiently concentrated solution of laminarin B.B.2. but a further solution, heated at 100° for 18 hr., showed no increase in depolymerisation.

The extraction of sample B.B.2. involved the heating of the seaweed with dilute hydrochloric acid (pH 3.4; 55° for 1 hr.). It was stated that no formation of oligosaccharide could be detected when laminarin B.B.2. was re-subjected to the conditions of extraction. Since laminarin B.B.2. has now been shown to contain traces of oligosaccharides, treatment with dilute hydrochloric acid was applied to sample 6 which had been extracted without the use of acid and which was oligosaccharide-free. No degradation could be detected however, thus the use of dilute hydrochloric acid during extraction probably has little effect on the average

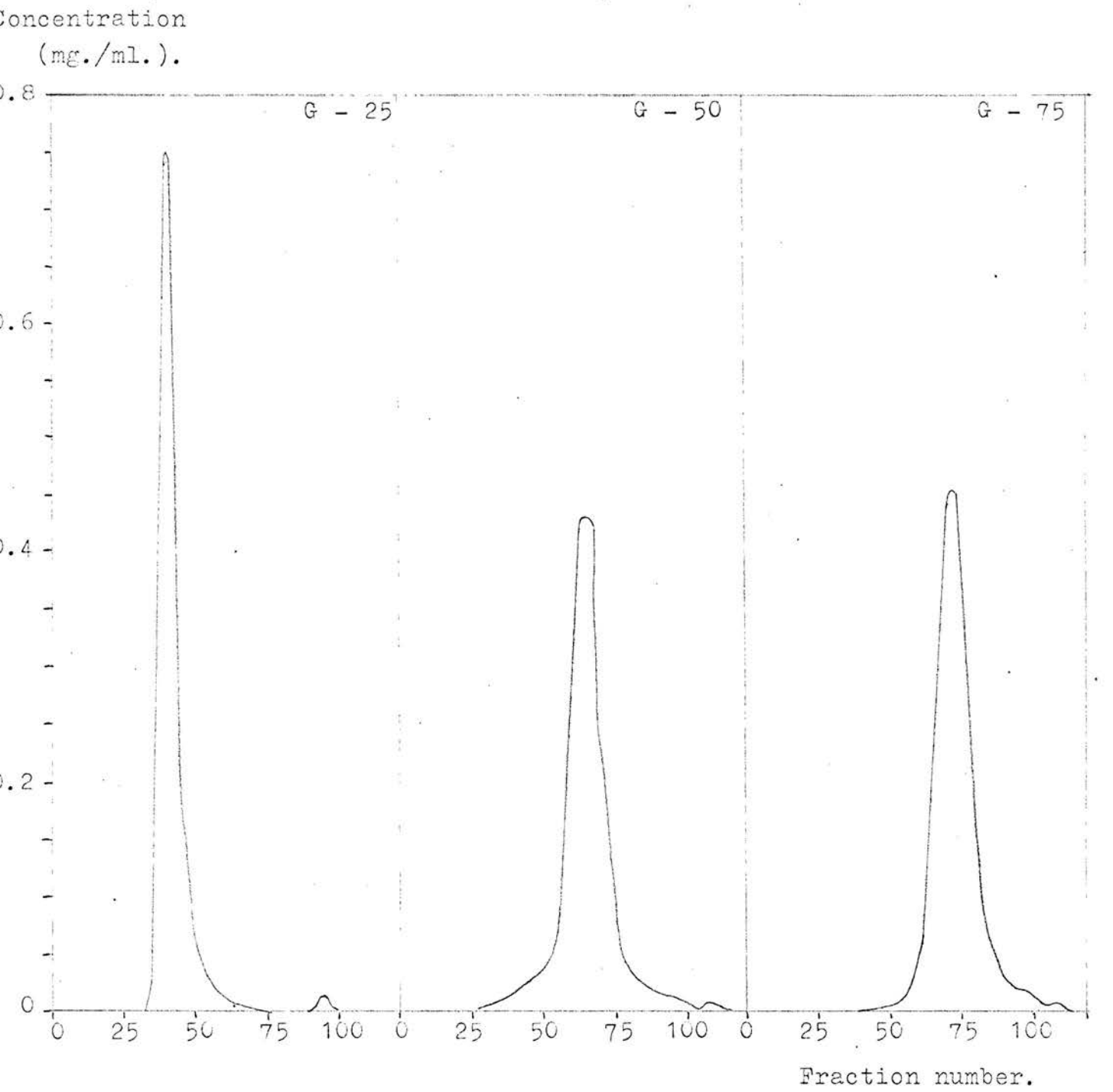


Fig. 22. GEL-FILTRATION OF LAMINARIN.

D.P. of laminarin.

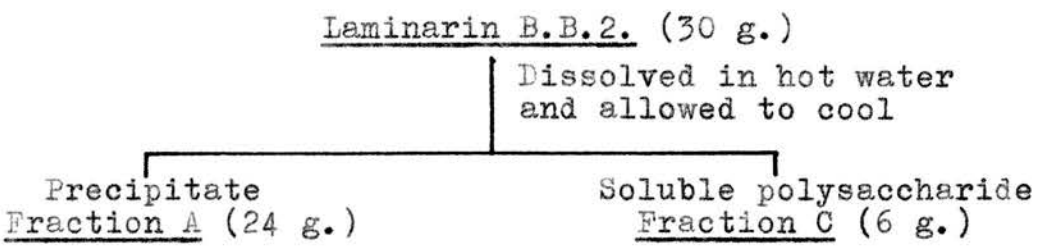
b) Gel-filtration.

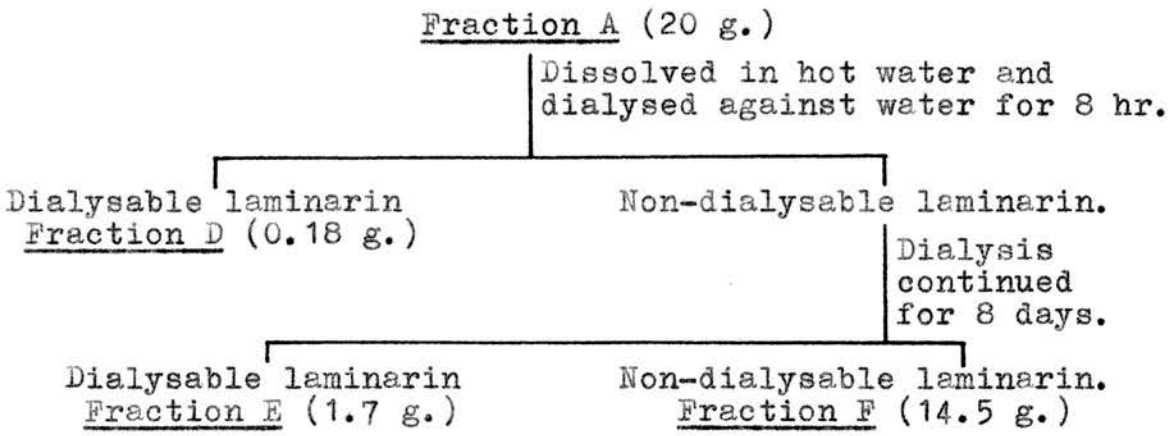
In an attempt to detect any gross heterogeneity, sample B.B.2 was fractionated on columns of Sephadex G-25, G-50 and G-75 (medium grades). Only small amounts of material (ca. 25 mg.) could be used owing to the low solubility of this sample. Elution was followed by the phenol/sulphuric acid method, and the total recovery was checked in two cases and found to be substantially quantitative.

All three types of Sephadex produced one main peak, followed by a small peak, corresponding to about 0.5% of the recovery, which was observed by paper chromatography to contain glucose (Fig. 22). The source of this trace amount of glucose is not known. These preliminary experiments suggest that, on a weight basis, sample B.B.2. is not very heterogeneous.

Fractionation by spontaneous precipitation and dialysis.

Dr.F.B.Anderson fractionated laminarin B.B.2. according to the following scheme:





The above fractions have been examined. Purity was checked by measurement of the glucose content. The release of formaldehyde on periodate oxidation was used, at 2°, to estimate the mannitol content and, after 2-3 days' oxidation at room temperature, to indicate approximate values for the D.P. The content of reducing glucose residues was investigated further by determination (in duplicate) of the reducing power.

Results for laminarin B.B.2 are given for comparison:

Fraction	A	C	D	E	F	Laminarin B.B.2.
Glucose content (%)	93	91*	-	94	96	94
Mannitol content (%)	2.4	3.1	3.1	2.8	2.3	2.4
Approximate D.P.	27	26	22	22	28	27
Apparent content of reducing glucose residues (%)	1.53 1.48	1.25 1.24	- -	1.37 1.39	1.46 1.47	1.45 1.43

* Fraction C had an ash content of 1.4% (cf. 0.6% for laminarin B.B.2.)

Some heterogeneity is apparent from these results. Fraction C which remained in solution after spontaneous precipitation has a similar molecular weight to the precipitated material but an appreciably higher mannitol content and, consequently, a lower reducing power. In view of the association between solubility and degree of branching found earlier, this finding would associate branching with molecules terminated by mannitol, in agreement with the results of Anderson et al.[42] from lime water degradation.

Fractions D and E, which were furnished by dialysis, appear to have lower values of D.P. than the parent Fraction A, as would be expected. The rate of dialysis seems also to be associated with a higher mannitol content. This is confirmed by the fact that, despite its lower D.P., Fraction E has also a lower reducing power than Fraction A. Molecules terminated by mannitol therefore include those of molecular weight lower than the average.

Experimental

Periodate oxidation of laminarin samples.

a) Release of formaldehyde at 2°.

Portions (10 ml.) of solutions of sample S.L.5.

(77.9 mg.; 25 ml.) and sample 5 (86.2 mg.; 25 ml.) were oxidised in the manner described for the previous samples (p. 78). The release of formaldehyde (mol./ anhydrohexose residue) was:

Time (hr.)	S.L.5.	5*
1	0.029	0.029
6	0.028	0.030
24	0.030	-

*Sampled at 1.5 and 5.5 hr.

b) Release of formaldehyde at room temperature.

i) Portions (10 ml.) of solution of sample B.B.2. (88.4 mg.; 25 ml.), sample S.L.5. (90.7 mg.; 25 ml.), Borax sample (88.5 mg.; 25 ml.) and Smith's sample (87.5 mg.; 25 ml.) were oxidised as previously described (p.32). The results are shown on p. 102.

ii) The 16 day oxidation was carried out with portions (10 ml.) of solutions of sample B.B.2. (82.7 mg.; 25 ml) and sample 6 (89.4 mg.; 25 ml.). To ensure an adequate excess of periodate, 1 ml., instead of 0.5 ml., of 0.3 M sodium metaperiodate was added. Normal procedure was then adopted except that M sodium sulphite was used. The results are shown in Fig. 19.

Preparation of laminaritol from sample 6.

A solution (150 ml.) of sample 6 (5 g.) was treated

with potassium borohydride solution (1.5 g.; 15 ml.). After 18 hr. a further portion of potassium borohydride solution (1.0 g.; 10 ml.) was added. After a total of 42 hr., the solution was neutralised with glacial acetic acid and ethanol (6 vol.) added. The precipitate was washed twice with methylated spirits and dissolved in water (70 ml.). After reprecipitation with ethanol (6 vol.), the laminaritol was washed with methylated spirits and ether and dried. Yield: 4.8 g. Glucose content (by cuprimetric titration of a hydrolysate): 92%.

Oxidation of laminaritol samples at room temperature.

a) Release of formaldehyde.

Portions (20 ml.) of solutions of laminaritol B.B.2. (38.1 mg.; 50 ml.) and laminaritol 6 (42.8 mg.; 50 ml.) were treated with 0.03 M sodium metaperiodate (1 ml.). Samples (1 ml.) were added to 0.1 M sodium sulphite (0.5 ml.). Difficulty was experienced in reducing blank values as the polysaccharide concentration was so low as to preclude efficient ethanolic precipitation. Ethanol (5 ml.) was used and a portion (2 ml.) of the supernatant treated with the chromotropic acid reagent (20 ml.), and thiourea solution (2 ml.). Optical densities were determined using 4 cm. cells. (Fig.20).

b) Reduction of periodate.

In order to confirm that an adequate excess of periodate was present, portions (2 ml.) were diluted to 50 ml. and the optical densities at 222.5 mμ determined. The original laminaritol solutions had optical densities of 0.037 and 0.190 respectively; a small correction of 0.008 was therefore applied in the case of laminaritol 6. The reduction of periodate (mol./anhydrohexose residue) was:

Time (hr.)	Laminaritol B.B.2.	Laminaritol 6.
2	0.13	0.13
6	0.16	0.17
cf. periodate supplied	0.32	0.28

Periodate oxidation of laminarin samples at 2°.

a) 7.5 mM Periodate.

i) Release of formic acid.

It was confirmed by titration of portions (20 ml.) of deionised water, a solution of laminarin B.B.2. (155.1 mg.; 100 ml.) and a solution of laminarin 6 (177.2 mg.; 100 ml.) that the polysaccharides were accurately neutral. Each portion required 0.04 ml. of 0.0095 N barium hydroxide to give pH 6.3

Laminarin B.B.2. (401.0 mg.) and sample 6 (446.3 mg.) were dissolved in water (ca. 200 ml.) and the solutions

were treated, at 2°, with 0.1875 M sodium metaperiodate (10 ml.) and water to 250 ml. Samples (25 ml.) were added to 12.4% ethylene glycol (5 ml.) and titrated in the usual way with 0.0095 N barium hydroxide (Fig. 21).

ii) Reduction of periodate.

Samples (2 ml.) were diluted to 250 ml. for estimation. It was observed that a solution of laminarin B.B.2. (155.1 mg.; 100 ml.) and a solution of laminarin 6 (177.2 mg.; 100 ml.) had optical densities at 222.5 mμ of 0.086 and 0.812 respectively. A correction of 0.007 was therefore made in the latter case. The reduction of periodate (mol./anhydrohexose residue) was:

Time (hr.)	Sample B.B.2.	Sample 6.
1	0.105	0.124
6	0.163	0.185
19	0.206	0.256
43	0.223	0.304
67	0.232	0.328
100	-	0.344
cf. periodate supplied	0.76	0.68

b) 75 mM Periodate.

Release of formic acid.

Solutions (ca. 130 ml.) were prepared with laminarin B.B.2. (407.1 mg.) and laminarin 6 (414.7 mg.). The solutions were treated, at 2°, with 0.1875 M sodium

metaperiodate (100 ml.) and water to 250 ml. Samples (25 ml.) were added to ethylene glycol (ca. 2 ml.) before titration, during which barium iodate (identified with sulphurous acid and carbon tetrachloride) was precipitated (Fig. 21).

Paper chromatography of laminarin samples.

Eight applications to No. 3MM paper of laminarin solutions (25 mg.; 0.5 ml.) were made. Aniline phthalate and silver nitrate reagents were used.

Laminarin B.B.2. (ca. 100 mg.) in water (1.5 ml.) was heated at 100° for 18 hr. and chromatographed as above.

Examination of conditions of extraction.

Acid of pH 3.5 was obtained by diluting "Analar" hydrochloric acid (1 part to 30,000 parts). Laminarin 6 (25 mg.) was dissolved in the dilute acid (20 ml.) at 55-60° and this temperature was maintained for one hr. The solution was cooled, neutralised with silver carbonate, evaporated to 0.5 ml. and examined by paper chromatography.

Gel-filtration of laminarin B.B.2.

Columns (4 x ca. 40 cm.) were prepared with

Sephadex G-25 (100 g.), G-50 (70 g.) and G-75 (50 g.). Portions (10 ml.) of laminarin B.B.2. solutions (ca. 25 mg.: 68.5 mg.; 25 ml.: 62.1 mg.; 25 ml. respectively) were applied and eluted with a flow rate of one fraction (4.54 ml.) per 5 min. Estimated recoveries from the last two columns were 95% and 100%.

Fractionation of laminarin B.B.2. (by Dr.F.B.Anderson).

Laminarin B.B.2. (30 g.) was dissolved in hot water (2 l.) and the solution was allowed to cool. After 2 days the precipitate (24 g.; Fraction A) was removed by centrifugation. After a further 2 days another precipitate (0.3 g.; Fraction B) was removed and the polysaccharide remaining in solution was recovered (6 g.; Fraction C).

Fraction A (ca. 20 g.) was dissolved in hot water (500 ml.) and the solution was dialysed at 35° against water (5 l.). After 8 hr., the dialysate was replaced with fresh water and the temperature maintained at 35° for 8 days. Polysaccharide was recovered from both dialysates and purified by reprecipitation with ethanol from aqueous solution giving Fraction D (0.18 g.) and Fraction E (1.7 g.). The non-dialysable material was recovered (14.5 g.; Fraction F).

Examination of Fractions.

a) Glucose content.

Cuprimetric titration of hydrolysates was employed.

b) Release of formaldehyde on periodate oxidation at 2° and at room temperature.

Portions (10 ml.) of solutions (ca. 90 mg.; 25 ml.) were oxidised as previously described.

c) Reducing power.

Samples (ca. 60 mg.) were dissolved in hot water (5 ml.). The solutions were cooled to room temperature before the addition of the copper reagent (5 ml.). A heating period of 20 min. was employed.

SUMMARY

I. Only minute quantities (ca. 0.2%) of mannose have been detected in hydrolysates of laminarin samples. It is considered that this sugar, in common with fucose, arises from contaminating carbohydrate and is not structurally significant. The possible formation of mannose as an artefact by the methods used by Smith *et al.*[48] and Chesters *et al.*[49] has been examined by control experiments but this has failed to explain their results.

II. 1-Substitution, as opposed to 1,2- disubstitution, of the mannitol residues in laminarin has been established by two procedures. Selective periodate oxidation demonstrated the release of three mol. of formic acid from each mannitol residue, and, in contrast with the results of Goldstein *et al.*[45], ethylene glycol was isolated from the hydrolysis products of laminarin polyalcohol. Gas-liquid chromatography has also revealed a component of methylated laminarin with retention times identical to those of 2,3,4,5,6-pentamethyl mannitol.

III. Mild acid hydrolysis of laminarin polyalcohol, to cleave acetal but not glucosidic linkages, has furnished no evidence for the presence of 1,6- inter-residue, as

opposed to inter-chain, linkages in laminarin. Oligosaccharide fragments could not be detected in such hydrolysates and gel-filtration further demonstrated, by the complete resolution of the degraded polysaccharide material from a reference pentasaccharide marker, that small fragments were absent. Absence of 1,6- inter-residue linkages from a sample of methylated laminarin was confirmed by the failure to detect 2,3,4-trimethyl glucose as a constituent sugar.

IV. Differences between individual laminarin samples have been studied by periodate oxidation, and heterogeneity within one sample has also been examined. The mannitol contents of six laminarin samples lay within the range $2.9 \pm 0.5\%$. The degrees of polymerisation of samples B.B.2. and 6 were 24 and 28, the proportions of molecules terminated with mannitol being 57% and 75%, and the numbers of branch points per molecule for these samples were 0.3 and 1.6. The figure of 0.3 for sample B.B.2. has been confirmed by estimation of the glycerol content of the derived polyalcohol. The marked difference between the degrees of branching suggests that this feature may be associated with spontaneous precipitation from aqueous solution, the latter being a property of sample B.B.2. but not of sample 6.

Sample B.B.2, in contrast with other samples,

contained minute traces of laminaridextrins and was examined in more detail. Fractionation by spontaneous precipitation furnished a soluble fraction with a slightly enhanced mannitol content. Dialysis of the insoluble fraction produced dialysable material, again with an enhanced mannitol content but also with a slightly reduced degree of polymerisation. Gel-filtration of sample B.B.2. revealed no gross hetero-geneity.

STUDIES ON LICHENIN

INTRODUCTION

Detailed structures have been assigned to only three lichen polysaccharides; these are pustulan from Umbilicaria pustulata, and isolichenin and lichenin from Cetraria islandica. The latter lichen also elaborates complex heteropolysaccharides[147] about which little is known except that they contain glucose, galactose, mannose and uronic acid. A similar complex mixture of heteropolysaccharides, which appear to be highly branched, has been obtained from Cladonia alpestris.[148]

Drake[149], and Lindberg and Macpherson[150] have shown that pustulan is an unbranched β-1,6- linked glucan. Isolichenin[151,152] is a linear α-glucan containing both 1,3- and 1,4- linkages, the former occuring to the extent of 55-60%. Some regular pattern of linkage distribution may occur but strict alternation of 1,3- and 1,4- linkages, as in nigeran[153], has been excluded. The linear glucan, lichenin, similarly contains both 1,3- and 1,4- linkages, the former constituting 70% of the total, but the linkages in this polysaccharide possess the β-configuration. The structural features of lichenin will be discussed later in more detail.

It is interesting to consider the symbiotic nature of lichens, each of which consists of an alga and a fungus. Smith and Montgomery[154] have speculated that the lichen glucans arise from the algal component and that the more complex mixtures of heteropolysaccharides derive from the fungus, but little is known about the carbohydrate mechanism of lichens. A recent experiment[155] has shown, however, that when the lichen Peltigera polydactyla photosynthesises in the presence of ^{14}C-bicarbonate or ^{14}C-glucose much of the fixed carbon was initially incorporated into mannitol - a result similar to that obtained by Bidwell et al.[156] with the brown alga, Fucus vesiculosus. It was also possible to separate the algal layer of the lichen thallus from the medulla which is composed entirely of fungal hyphae. By this means it was shown that fixation of carbon by the lichen occurred more rapidly in the algal layer than in the medulla and further that, in the dark, the radioactivity of the medulla continued to rise for a few hours at the expense of that of the algal layer. Transfer of material to the fungus was therefore indicated.

The algal components of lichens belong to widespread and free-living genera of Chlorophyceae and Cyanophyceae. The alga present in the lichen Collema tenax, for example, is classified as a Nostoc. It is apparent

that the carbohydrates synthesised by these algae are varied. Examples of green algae include Chlamydomonas spp. which produce polysaccharides[157] containing galactose, glucose, arabinose, xylose and smaller amounts of other sugars. Dunaliella bioculata furnishes a starch[158]. Many carbohydrate components have been isolated from Chara[159] and Nitella[160], but the affinities of the Charales are obscure. Among the blue-green algae, Oscillatoria spp.[161] synthesise an amylopectin and both Anabaena cylindria[162] and Nostoc spp.[161] produce complex mixtures of polysaccharides which contain some five neutral sugars together with uronic acid.

The fungal component is almost invariably an Ascomycete which group includes such genera as Saccharomyces, Penicillium and Claviceps. Polysaccharides isolated from yeasts include typical glycogens[137,163], branched α-mannans containing varying proportions of 1,2- , 1,3- and 1,6- linkages[164,165,166,167] and one which appears to contain 1,2- and 1,6- linkages only[168]. β-Glucans have also been isolated and three structures for these have been advanced. Bell and Northcote[169] found a highly branched structure containing both 1,3- and 1,2- linkages. Linkage analysis[170] of a different sample produced no evidence for branching and only 1,3-

and 1,6- linkages were detected. On the other hand, Bishop *et al.*[168], using periodate oxidation and methylation, found a highly branched, 1,3- and 1,6- linked polymer which contained a preponderance of 1,6- linkages, in contrast to the 10-20% present[171] in the previous sample. The yeast-like fungus *Pullularia pullulans* has provided an α-glucan, a β-glucan and an acidic heteropolysaccharide[172] containing mannose, glucose and galactose. Structural studies[173,174] on the α-glucan have established the presence of a repeating sequence of 1,6- linked maltotriose units, but the presence of a small proportion of 1,3- linkages has also been claimed[145]. It is stated[173 b] that the β-glucan possesses a chain of 1,3- linked glucose residues, of which two out of three carry a further glucose residue in position 6. A β-glucan, less branched but of generally similar structure is also elaborated by *Claviceps purpurea*[175].

It is apparent, from these few examples, that there is no *a priori* reason why the lichen fungi should not synthesise glucans. The symbiotic relationship can, however, be expected to influence the physiology of the organisms involved. It would therefore be of particular interest to culture the algal component of, for example, *Cetraria islandica* to determine whether it

could be persuaded to produce lichenin.

The present work concerns the fine structure of lichenin, and the more important evidence on which this is based will be presented here. Early work showed that complete acid hydrolysis produced glucose only, and enzymic hydrolysis to glucose was also observed. Other enzymic degradations furnished cellobiose and this disaccharide was also obtained by acetolysis. 2,3,6-Trimethyl glucose was established as the predominant product of methylation analysis.

In 1939, Carter and Record[176] studied the osmotic pressure of solutions of many acetylated and methylated polysaccharides. The observed D.P. of lichenin samples ranged from 50 to 400. The presence of 2,3,4,6-tetramethyl glucose in methylated lichenin was also noted, and the proportion of this component pointed to a C.L. of the same order of magnitude as the D.P. This was in marked contrast to results for glycogens which typically indicated a D.P. of 3,000 and a C.L. of 12. A linear structure for lichenin is therefore possible.

An analysis was carried out by Hess and Lauridsen[177] on a fully methylated sample of lichenin. The proportion of tetramethyl glucose indicated a C.L. of 115, and very little dimethyl glucose was found which substantiated the linear structure. A further

methylation analysis by Meyer and Gürtler[178] indicated a C.L. of 150-200. They, however, made the important observation that the trimethyl glucose fraction was a mixture of both 2,3,6- and 2,4,6-trimethylglucose. By periodate oxidation of unmethylated lichenin it was deduced that 1,4- linkages accounted for 74% of the total. Meyer and Gürtler also mention that lichenin appeared to possess a methoxyl content of 0.34-0.38%, corresponding to 2% of a monomethyl glucose. This has never been confirmed and no hexose other than glucose has been detected in a hydrolysate of lichenin. Boissonnas[179] applied his method of reduction followed by p-phenyl azo-benzoylation to the trimethyl glucose fraction and found 68% of 2,3,6-trimethyl glucose, in substantial agreement with the results of Meyer and Gürtler.

No further work was reported until 1957, when Chanda, Hirst and Manners[151] confirmed that 70% of the linkages were 1,4- by periodate oxidation and by methylation analysis; the latter also indicating a C.L. of 62. The presence of a trace only of dimethyl glucose confirmed the unbranched nature of the polysaccharide. Partial acid hydrolysis of lichenin furnished glucose, cellobiose, laminaribiose and higher oligosaccharides but, after periodate oxidation followed

by reduction with borohydride, partial acid hydrolysates no longer contained laminaribiose. Few, if any, sequences of two or more adjacent 1,3- linkages are therefore present in lichenin.

A large-scale partial acid hydrolysate of lichenin (50 g.), by Peat, Whelan and Roberts[180], furnished the following fragments:

Sugar	Yield (g.)
Glucose	16.3
Cellobiose	7.46
Laminaribiose	1.00
Cellotriose	4.09
3-O-β-Laminaribiosyl glucose	0.74
4-O-β-Cellobiosyl glucose	1.57

The structure of the last two trisaccharides provided convincing proof of the presence of both 1,4- and 1,3- linkages in the same polysaccharide molecule. The absence of two of the five possible trisaccharides, viz. laminaritriose and 3,4-diglucosyl glucose, is consistent with previous observations that sequences of adjacent 1,3- linkages and branching do not occur to any appreciable extent. Examination of the tetrasaccharide fraction for cellotetraose and 4^2-O-β-laminaribiosyl laminaribiose was unsuccessful and it was therefore deduced that 1,4- linkages occur largely in pairs, rather than in larger sequences or in isolation. These

findings prompted the suggestion that the structure was that of repeating 1,3- linked cellotriose units. This would require 67% of 1,4- linkages, in fair agreement with previously observed results. Peat and his co-workers reported two further determinations of the proportion of 1,4- linkages; 72.5% by periodate oxidation and 69% by comparison of the optical rotations of cellulose and lichenin in the presence of cuprammonium ion. It may be noted that, if a substantial proportion of isolated 1,4- linkages were present, these results would imply the presence also of sequences of more than two such linkages.

Further work on lichenin has been of an enzymic nature. Cunningham and Manners[181] showed that an enzyme system containing laminarinase activity, but not cellodextrinase, cellobiase or laminaribiase activities, degraded lichenin to a trisaccharide, 3-O-β-cellobiosyl glucose, together with small amounts of glucose, laminaribiose and tetrasaccharide. Laminarinase does not, therefore, cleave the 1,3- linkages but cleaves instead the glucosidic linkage of a 3- substituted glucose residue. Thus, in the present example, the linkages which were hydrolysed would be largely - possibly entirely - of the 1,4- configuration. These findings were confirmed by Perlin and Suzuki[182] who

suggested that the presence of a few isolated 1,4- linkages could account for the formation of small amounts of laminaribiose.

Perlin and Suzuki extended this work, however, by the use of a cellulase which was found to degrade lichenin to 4-O-β-laminaribiosyl glucose. Small amounts of cellobiose and two tetrasaccharides, 3^2-O-β-cellobiosyl cellobiose and 4^2-O-β-laminaribiosyl cellobiose were also isolated and it was stated that the presence of the two tetrasaccharides indicated the presence of sequences of more than two adjacent 1,4- linkages. It is apparent that cellulase, by analogy with laminarinase, cleaves the glucosidic linkage of 4- substituted glucose residues only. It is not clear, however, if cellulase is indifferent to the nature of the linkage which is actually cleaved; the apparent absence of cellotriose suggests that it may be reluctant to hydrolyse 1,3- linkages but no evidence concerning this point is offered. Perlin and Suzuki's claim that sequences of more than two adjacent 1,4- linkages exist appears to be based on the assumption that cellulase cannot hydrolyse 1,3- linkages. Should this assumption be false, the presence of 4^2-O-β-laminaribiosyl cellobiose needs no particular explanation and the previously suggested existence of a few isolated 1,4- linkages could account

for the formation of 3^2-O-β-cellobiosyl cellobiose.

Thus lichenin consists largely of repeating sequences of β-1,3- linked cellotriose units with the possible exception of occasional isolated 1,4- linkages and sequences of more than two adjacent 1,4- linkages. Mention must now be made of the β-glucan of oats[183,184] and that of barley[185], which have been studied by the methods outlined above. Only minor differences between lichenin and these polysaccharides have been observed. Estimates of the proportion of 1,4- linkages in barley β-glucan have ranged from 50% to 75%, and the presence of a low degree of branching in oat β-glucan has been suggested. No evidence for the presence of adjacent 1,3- linkages was obtained from a partial acid hydrolysis of oat glucan by Peat et al.[180] or from enzymic degradations of both glucans by Parrish, Perlin and Reese[186].

Smith and Montgomery[187], however, state that on periodate oxidation followed by reduction with borohydride and mild acid hydrolysis, oat glucan furnished, in addition to glucosyl erythritol, small quantities of laminaridextrin glycosides of erythritol up to laminaritetraosyl erythritol. These can have arisen only from sequences of up to four adjacent 1,3-linkages. Furthermore, Preece, Garg and Hoggan[188]

identified laminaritriose, by chromatography and electrophoresis, as a product of endo-enzymolysis of both barley and oat glucans. The possible presence of sequences of adjacent 1,3- linkages in lichenin must therefore be reconsidered and the present work consisted of an examination of lichenin by the procedure of Smith and Montgomery.

THE DISTRIBUTION OF 1,3- LINKAGES IN LICHENIN

A linear glucan consisting entirely of repeating sequences of β-1,3- linked cellotriose units should give rise exclusively to 2-O-β-glucosyl erythritol, erythritol and glycolaldehyde after periodate oxidation, borohydride reduction and mild acid hydrolysis. The presence of any sequences of 1,3- linkages would be revealed by the formation of erythritol glycosides of laminaridextrins containing glucose residues corresponding in number with the number of adjacent 1,3- linkages.

A sample of lichenin polyalcohol, prepared by Dr.G.A.Mercer, was subjected to mild acid hydrolysis (0.5 N sulphuric acid; 18 hr. at room temperature) and the neutralised hydrolysate was passed through a column of Amberlite IRA 400 OH^- resin to remove glycolaldehyde. Two fractions (each 6 bed vol.) were collected and both were found, by paper chromatography, to contain erythritol and two other non-reducing components, A and B, with R_G 1.0 and 0.75 in 4/1/5. The two fractions were combined and chromatographed on thick paper using 4/1/5. Substances A and B were eluted and rechromatographed using 4/1/5 and 6/4/3 which confirmed their purity (R_G 1.2 and 0.9 in 6/4/3). Paper electrophoresis also provided evidence of homogeneity; A and B had R_M

0.19 and 0.44 respectively using xylose as reference marker. On hydrolysis, both A and B gave glucose and erythritol.

The mild acid hydrolysis of lichenin polyalcohol was further investigated by sampling a hydrolysate at intervals. In view of the affinity of strongly basic resin for non-reducing, as well as reducing, carbohydrates, neutralised samples were examined directly by paper chromatography. Solvents 6/4/3 and 4/1/5 were found to eliminate sufficiently interference by glycolaldehyde. A trace of glucose was detected after hydrolysis with 0.25 N sulphuric acid for 43 hr. at room temperature.

Lichenin polyalcohol was therefore hydrolysed for 20 hr. with 0.25 N sulphuric acid. Chromatography (4/1/5) showed that the hydrolysate contained, in addition to erythritol, A and B, another non-reducing substance, C, with R_G 0.45 and traces of components of lower mobility. The mixture was resolved into four fractions, A, B, C and the remaining trace components, D. Rechromatography in 4/1/5 indicated that A,B and C were pure and, on electrophoresis in the manner used for A and B, C had R_M 0.35 (periodate/permanganate reagent). On complete acid hydrolysis, C furnished glucose and erythritol.

It was inferred that B, the largest fraction

obtained, was probably 2-O-β-glucosyl erythritol. Component C, from its chromatographic mobility, resembled a trisaccharide and could be 2-O-β-laminaribiosyl erythritol. A portion of C was methylated, methanolysed and examined by gas-liquid chromatography (polyphenyl ether column). Tetramethyl glucose, 2,4,6-trimethyl glucose and two more volatile components with relative retention times of 0.30 and 0.41 were observed. The presence of only one more volatile component viz. 1,3,4-trimethyl D-erythritol would be expected and C was therefore further investigated by partial acid hydrolysis.

A preliminary experiment with 1-O-β-laminaribiosyl mannitol showed that hydrolysis with 0.2 N sulphuric acid for 25 min. at 100° produced laminaribiose, a larger amount of glucosyl mannitol and left a substantial amount of the original compound unhydrolysed (R_G values in 6/4/3 of 0.84, 0.56 and 0.39). Under the same conditions, substance C was completely hydrolysed. Chromatography (4/1/5) showed that the products were 2-O-β-glucosyl erythritol (R_G 0.75), a substantial amount of erythritol and a little glucose. Laminaribiose (R_G 0.58) could not be detected. Chromatography in 6/4/3 showed that no material with the mobility of substance A was produced. On partial acid hydrolysis,

fraction D, which contained the components of lower mobility, behaved in a manner identical to that of fraction C. The failure to detect laminaridextrins indicated that sequences of 1,3- linkages cannot occur in lichenin.

The extreme ease of acid hydrolysis of fractions C and D suggested the presence of residual acetal, as well as glucosidic, linkages, and this was confirmed by the subsequent detection, chromatographically, of glycolaldehyde in hydrolysates of these fractions. In view of this and the fact that the currently accepted structure of lichenin does not account for the formation of the component with chromatographic mobility greater than that of 2-O-β-glucosyl erythritol, fraction A was subjected to partial acid hydrolysis. In addition to providing 2-O-β-glucosyl erythritol, erythritol and a trace of glucose, a substantial amount of non-reducing material of unchanged chromatographic mobility remained. This is unusual in that two of the products, glucosyl erythritol and glucose, have a lower chromatographic mobility than substance A (R_G values in 6/4/3 of 0.9, 1 and 1.2 respectively), and also in that an unidentified, glucosidically linked compound is apparently present.

Additional evidence for the presence of residual acetal linkages was provided by determination of the

ratio of erythritol to glucose for fractions A,B andC. Hydrolysates were chromatographed and erythritol and glucose were eluted and estimated by the release of formaldehyde on periodate oxidation and by the phenol/ sulphuric acid method respectively. The results were 1.5, 1.2 and 1.9, indicating that an excess of erythritol was present. Despite the homogeneity displayed on chromatography and electrophoresis, it is unlikely that fraction A, at least, is homogeneous.

To ensure complete hydrolysis of acetal linkages, lichenin polyalcohol was subjected to partial acid hydrolysis at 100°. The products were 2-O-β-glucosyl erythritol, glucose, a non-reducing component with the mobility of fraction A (R_G,in 18/3/1/4,1.2) and erythritol. 2-O-β-Laminaribiosyl erythritol was not available for comparison but chromatography (6/4/3) failed to detect any material of lower mobility than R_G 0.8,which corresponded to the tailing edge of the 2-O-β-glucosyl erythritol component. The presence of 2-O-β-laminaribiosyl erythritol is therefore most unlikely.

Experimental.

Preparation of lichenin polyalcohol (by Dr.G.A.Mercer)

A portion (10 g.) of lichenin, extracted by

Dr.F.B.Anderson[99], was shaken in the dark for 100 hr. with sodium metaperiodate (30 g.) in water (500 ml.). The oxopolysaccharide was recovered by centrifugation, washed several times with water and suspended in water (200 ml.) to which was added slowly with stirring a solution of potassium borohydride (2.0 g.; 50 ml.). After 24 hr., a further portion (1 g.) of potassium borohydride was added. After a total of 29 hr., the solution was neutralised with glacial acetic acid to pH 7 and ionic material was removed by electrodialysis. The solution was freeze-dried to yield lichenin polyalcohol.

Mild acid hydrolysis of lichenin polyalcohol.

Lichenin polyalcohol (50 mg.) was dissolved in hot water (5 ml.). The solution was cooled to room temperature and N sulphuric acid (5 ml.) added. After 18 hr., the solution was neutralised and passed through a column (34 ml.) of Amberlite IRA 400 OH^- resin. Two fraction (each 200 ml.) were collected and combined after paper chromatographic examination. Components A and B were then isolated by thick paper chromatography and examined. Silver nitrate was used to reveal the components after electrophoresis while aniline phthalate was used for the reference markers.

A hydrolysate (50 mg. in 20 ml. of 0.25 N sulphuric acid) prepared similarly to the above was sampled at 3.5, 7, 19 and 43 hr. Samples (5 ml.) were neutralised with barium carbonate before paper chromatography.

A hydrolysate of lichenin polyalcohol (100 mg.) in 0.25 N sulphuric acid (20 ml.) was neutralised after 20 hr. and fractions A, B, C and D were isolated.

Methylation of substance C.

A portion (glucose content of 0.7 mg. by the phenol/sulphuric acid method) of substance C was methylated[189] with methyl iodide (0.2 ml.) and silver oxide (0.2 g.) in dimethyl formamide (0.2 ml.) with continuous mixing on rollers for 18 hr. After extraction of the product with chloroform, the latter was removed by evaporation and the product was treated with methyl iodide (0.2 ml.) and silver oxide (0.2 g.) as above for a further 24 hr. The product was again extracted with chloroform and the evaporated extract subjected to a high vacuum at room temperature for 40 min. to remove dimethyl formamide. The residual material was methanolysed in a sealed tube for 5 hr. and the neutralised methanolysate examined by gas-liquid chromatography.

Determination of erythritol/glucose ratios.

The reliability of the procedure was first confirmed with standard solutions. A 0.096% solution of erythritol (24.1mg.; 25 ml.) was prepared and a portion (1 ml.) diluted to 50 ml. A portion (4 ml.) of the diluted solution was treated with 0.01 M sodium metaperiodate (0.5 ml.) and samples (1 ml.) were withdrawn after 1 and 2 hr. and added to 0.05 M sodium sulphite (0.5 ml.). Estimation of formaldehyde, completed using chromotropic acid reagent (10 ml.) and thiourea solution (1 ml.), indicated an original erythritol concentration of 0.093%. A 0.136% solution of glucose (33.9 mg.; 25 ml.) was prepared. A portion (0.5 ml.) was added to water (10 ml.) for estimation by the phenol/sulphuric acid method and a concentration of 0.139% determined for the original solution.

Portions (10 ml.) of both original solutions were combined, evaporated to a small volume and the mixture chromatographed in duplicate using 4/1/5. The regions (ca. 24 sq.cm.) containing glucose were removed and soaked in water (10 ml.) for 0.5 hr., paper blank determinations being prepared appropriately. The eluates were filtered through glass wool and the glucose recoveries estimated at 0.345 and 0.115 mg. Erythritol was eluted in a similar manner and portions (2 ml. and

6 ml., respectively) of the eluates were adjusted to 4 ml. and oxidised as above. The recoveries were 0.236 and 0.075 mg., leading to erythritol/glucose ratios of 1.01 and 0.98 which agree well with 1.02 derived from the average of the previous figures.

Hydrolysates of A, B and C were chromatographed similarly and portions of the glucose eluates, suitably concentrated when necessary, were analysed, giving recoveries of 0.22, 0.62 and 0.14 mg. respectively. The corresponding erythritol recoveries were 0.224, 0.501 and 0.183 mg.

Partial acid hydrolysis of lichenin polyalcohol.

Lichenin polyalcohol (25 mg.) was heated with 0.1 N sulphuric acid (4 ml.) for 30 min. at 100°. The hydrolysate was neutralised with barium carbonate.

SUMMARY

The absence of erythritol glycosides of laminaridextrins in partial hydrolysates of lichenin polyalcohol has shown that the 1,3- linkages present in lichenin occur entirely in isolation rather than as sequences. Partial acid hydrolysates, however, contained a glucosidically-linked non-reducing component with a chromatographic mobility greater than that of glucose in 6/4/3 and 18/3/1/4. This has not yet been identified, and consideration of the currently accepted structure of repeating 1,3- linked cellotriose units for lichenin suggests no manner in which it might arise.

The persistence of a substantial proportion of acetal linkages after hydrolysis of lichenin polyalcohol at room temperature with 0.25 N sulphuric acid for 20 hr., showed that these linkages are more stable than had been supposed, e.g. by Unrau[60] who suggested hydrolysis with 0.1 N sulphuric acid for 10 hr. at room temperature.

APPENDIX A: THE ISOLATION OF LAMINARIN

The occurence of laminarin in many littoral and sub-littoral brown seaweeds has been extensively studied[52,190]. Among the Laminaria spp. laminarin is found only in the frond, as opposed to the stipe, and its distribution throughout the former is not uniform[191]. The laminarin content tends to decrease with increasing depth of immersion[192].

Marked seasonal variation of the laminarin content is also observed, laminarin comprising from 0-30% of the dry weight of Laminaria fronds, with the maximum occurring usually in the autumn. Inspection of Black's results shows that this variation may be rather irregular, e.g. the maximum laminarin content in L.cloustoni occurred in December 1945 but in September of the following year. The dry weight and ash content of the frond show a maximum and a minimum, respectively, which correspond closely with the maximum laminarin content. Furthermore, by plotting the former two variables against the laminarin content, a reasonably good association is revealed. A rough indication of the laminarin content can therefore rapidly be obtained from the dry weight and ash content, as an alternative to the estimation procedure of Cameron, Ross and

Percival[193]. Black's results suggest that, to ensure a laminarin content of 15%, the dry weight contents of L.saccharina and L.digitata shoulc be at least 14.5% and 16% and that the ash contents should not be more than 25% and 21% respectively.

Extraction procedures have been studied in detail by Black et al.[194] who finally adopted the use of dilute hydrochloric acid (pH 2.4) at 70° for 1 hr. Although β-1,3-glucosidic linkages appear to be little affected by this treatment, laminarin has been extracted, in the present work, without the use of acid to reduce the possibility of inadvertent degradation. Black et al. found that spontaneous precipitation of insoluble laminarin was largely independent of the pH of the solution, but Reid[195] has subsequently mentioned that, in the absence of acid, precipitation was delayed for 3-4 days.

Extraction of sample 5.

L.hyperborea fronds, harvested by Mr.E.Booth at Gairloch, were washed rapidly in fresh water to remove foreign material and batches (1.25 to 6 kg.) were minced (4 mm. plate) into 0.01 M mercuric chloride solution (0.5 parts by volume) at 70°. The mixture was stirred for 2 hr. at 70° and the extract then separated, with

some difficulty, by filtration through muslin and centrifugation. Extracts commonly had a pH of about 6.5. The residue was re-extracted twice with portions (0.25 parts by volume) of water at 70° for 0.5 hr. and the extracts were combined and stirred at room temperature for several hr. Little precipitation was observed even after the extracts had been stored at 2° for ten days.

Batches were also extracted with water and 0.125 N hydrochloric acid in place of 0.01 M mercuric chloride solution. Separation of the acid extract, which had pH 2.2, was readily accomplished but neither this nor the water extract yielded much laminarin. Precipitates were separated by centrifugation and purified by re-deposition from aqueous solution. Each sample was dissolved in a small volume of hot water and any insoluble material removed by centrifugation before the laminarin could reprecipitate. The highest yield was 32.4 g., after three reprecipitations, from a 6 kg. batch of weed which had been extracted with 0.01 M mercuric chloride solution. This crop was dissolved in hot water (300 ml.) and ethanol added with stirring to the cooled solution to give a final volume of 1,500 ml. The precipitate was washed with methylated spirits and ether, and dried. The product, referred to as sample 5,

amounted to 16.1 g. No mobile material could be detected by paper chromatography. A hydrolysate, chromatographed in 10/4/3, 18/3/1/4 and 9/1/1 with spray reagents 1,2 and 4, contained, apart from glucose, a little mannitol, and only traces of fucose and a component of lower mobility than glucose. Mannose, arabinose and xylose were absent.

Preliminary examination of L. saccharina and L. digitata.

In view of the very low yield obtained above, samples of L. saccharina and L. digitata were examined. Two plants of each species were collected from St. Abb's at low-water spring tides and the fronds were sampled for analysis. Portions (3 g.) were used for estimation of dry weight content, and portions (0.5 g.) of dried material were ashed.

	L. saccharina	L. digitata
16th August (1962)		
Dry weight (%)	13.8	14.5
16th September.		
Dry weight (%)	16.6	17.8
Ash (%)	24	25

The dry weight contents, which suggested a low laminarin content in August, showed a significant increase in September. Comparison of the September figures with Black's results indicated that, while the ash content of L. digitata was rather high, a substantial

laminarin content could be present in L. saccharina. The latter species was accordingly harvested at the next opportunity (15th October).

Extraction of sample 6.

The seaweed was washed rapidly in fresh water, about 5 hr. after being harvested, and the fronds (4.8 kg.) were divided into three batches, each of which was minced (3 mm. plate) into hot 0.01 M mercuric chloride solution (1.6 l.). The mixture was stirred at 70° for 2 hr. and strained through muslin. The residue was re-extracted twice with portions (1.2 l.) of hot water for 10 min. The extracts were combined and heated to 50° and centrifuges (Sharples supercentrifuge) while still warm. The solution (ca. 9 l.) was evaporated to 1,060 ml. in a cyclone evaporator and added to ethanol (4 vol.). Further additions of ethanol caused further precipitation; the amount of ethanol was therefore made up to 7 vol. and the total precipitate was washed with methylated spirits and ether, and air-dried. Yield: 320 g. (6.7 % of fresh weed) Ash content: 15%.

A hydrolysate contained glucose, as the main component, fucose, mannitol, and traces of mannose and xylose (chromatographic solvents and spray reagents as on p.145). Chromatography (10/4/3) of the unhydrolysed

material revealed free mannitol, and a component of low mobility which reacted with ninhydrin.

The crude laminarin (50 g.) was dissolved in water (600 ml.) and the solution was filtered to remove insoluble material and applied to a mixed-bed column consisting of Amberlite IR 120 H^+ resin (300 ml.) and Amberlite IR 45 OH^- resin (300 ml.). The eluate (1.5 l.) was collected, followed by two further fractions (each 300 ml.). The last two fractions were estimated (phenol/sulphuric acid method) to contain 0.18 g. and 0.144 g. of polysaccharide and were rejected. The eluate (1.5 l.), which was slightly acidic owing to the relatively less effective removal of anions such as free fucoidin acid, was evaporated for one hr., at a bath temperature of ca. 35°, to 700 ml. The pH fell from 2.4 to 2.1 during the evaporation but this acidity could have negligible effect during one hr. at 35°. Ethanol (5 vol.) was added slowly with stirring and the laminarin was coagulated by the addition of sodium chloride solution (0.5 g.; 3 ml.) and by the use of heat. The precipitate was washed and dried as before. Yield: 24.5 g. A residue of ca. 8 g. was obtained by evaporation of the ethanolic solution.

Comparison of hydrolysates of the precipitate and the soluble material showed that, while glucose was

still the predominant sugar in the soluble fraction, mannose and xylose were detectable only in the latter fraction, which was also greatly enriched in fucose. Free mannitol was still present in the precipitate, which was therefore redissolved in water (450 ml.) and reprecipitated with ethanol (4 vol.). The yield of washed and dried material was 23.6 g. The product, referred to as sample 6, contained no material mobile on paper chromatography, and a hydrolysate contained, apart from glucose and a little mannitol, only traces of fucose and a component of lower mobility than glucose.

APPENDIX B: FRACTIONATION OF POLYHYDRIC ALCOHOLS ON ANION-EXCHANGE RESIN

The chromatographic separation of sugars with ion-exchange resins has employed several techniques. The establishment of complexes of sugars with the borate ion led to the use of columns of strongly basic anion-exchange resin in the borate form which were eluted with solutions of borate[133,196]. It was then shown[197] that cation-exchange resin, in salt form and with water as eluant, effected fractionation in order of decreasing molecular size. An attempt[198] to use carbonate complex formation, in the manner of borate complex formation, also resulted in fractionation in order of decreasing molecular size. This result was obtained with the chloride form, as well as the carbonate form, of the resin, but fractionation was not observed with polystyrene resin which contained no ionic groups. This process therefore seems to require some form of bonding, and differs from gel-filtration. Partition chromatography, giving the more usual elution in order of increasing molecular weight, has been demonstrated[199] with strongly basic anion-exchange resin in the sulphate form and aqueous ethanol as eluant.

In the present work, strongly basic anion-exchange

resin in the hydroxide form was used to remove reducing carbohydrates from solution. A hydrolysate of laminarin polyalcohol, containing glucose, glycolaldehyde, arabitol, glycerol and ethylene glycol, was treated in order to obtain the last three components. It was shown (p. 80) that ethylene glycol was recovered quantitatively from the resin by elution with 3 bed vol. of water, and, by paper chromatography, that glycerol was similarly eluted. Fractionation of polyhydric alcohols on analytical grade (20-50 mesh) resin was not anticipated as the work described above generally indicated the need for long columns (ca. 70 cm.) of 200-400 mesh resin. Should any fractionation occur however, elution in order of decreasing molecular size could be expected on account of the results of Jones et al.[197] and Hough et al.[198]. The very low recovery of arabitol (see p. 74) suggested that marked fractionation in the reverse order had taken place and this has now been confirmed.

A mixture of ethylene glycol (48.6 μ mol.) and arabitol (23.5 μ mol.) was chromatographed on a column (1.7 x 15 cm.) of Amberlite IRA 400 OH^- resin. The column was eluted with deionised water and eight fractions were collected; the first four of 25 ml. and the last four of 50 ml. each. By estimation of the

release of formaldehyde on periodate oxidation in bicarbonate buffer, the recoveries were determined:

Fraction.	1	2	3	4	5	6	7	8
Alcohol (μ mol.)	17.3	28.0	8.00	4.73	7.35	4.22	1.99	0.85

Total recovery = 72.4 μ mol. cf. 72.1 μmol. used

Nine bed vol. of eluant was therefore required for complete elution. Full analysis of the fractions by measurement of the reduction of periodate was attempted but, under the conditions used (limited excess of periodate in bicarbonate buffer), unsatisfactory results were obtained. Paper chromatography, however, showed that fractions 4 to 8 contained only arabitol, a trace of which appeared in fraction 2. Furthermore, the recovery of arabitol in fractions 5-8 (fractions 1-4 amounting to 3 bed vol.) was 14.4 μ mol. which was 61% of the quantity used, and this proportion corresponds well with the apparent loss of arabitol from the hydrolysate of laminarin polyalcohol.

Dr.J.C.P.Schwarz suggested that the observed fractionation arose from the increase in acidity shown by the higher polyhydric alcohols. The pK values decrease steadily from ethylene glycol (14.24) to mannitol (13.50). The experiment was therefore repeated, using conditions more conducive to efficient

fractionation, in order to confirm the above work. A mixture of ethylene glycol, glycerol, erythritol, arabitol and mannitol was applied to a column (42 x 2.5cm.) of Amberlite C.G. 400 OH^- resin (100-200 mesh) and eluted using a slight linear concentration gradient [200] of ammonium carbonate. Fractions (25 ml.) were collected and an excellent fractionation was observed, fractions 7, 10, 15, 20, and 40 containing pure samples of the components from ethylene glycol to mannitol. No material could be detected in fractions 29 and 50; mannitol was therefore recovered completely in a pure state. The earlier fractions were not further investigated.

Since this work was done, Austin, Hardy, Buchanan and Baddiley[201] have reported the discovery that mixtures of glycosides were successfully fractionated during the removal of reducing carbohydrates, present as impurities, by strongly basic anion-exchange resin in the hydroxide form.

Experimental

Fractionation of glycol and arabitol on Amberlite IRA 400 OH^- analytical grade resin.

1.88% Ethylene glycol solution (2 ml.) was added to a portion (10 ml.) of arabitol solution (111.7 mg.; 25 ml.)

and the volume made up to 25 ml. A portion (2 ml.) of the mixture was applied to a column (1.7 x 15 cm.) and eight fractions (4 x 25 ml.; 4 x 50 ml.) were obtained. A portion (10 ml.) of each fraction was used for estimation, except in the case of fractions 1-3 where portions of 5, 1 and 2 ml., diluted to 10 ml., were used. 0.5 M Sodium bicarbonate (1 ml.) and 0.06 M sodium metaperiodate (1 ml.) were added, and samples (1 ml.) were withdrawn after 0.5 hr., treated with 0.1 M sodium sulphite (0.5 ml.), and the estimation was completed in the usual manner. 0.00447 Arabitol solution (10 ml.) was similarly oxidised and a virtually quantitative yield (1.94 mol.) of formaldehyde was observed under these conditions.

Estimation of the reduction of periodate was attempted using a portion (5 ml.) of each fraction, to which was added 0.5 M sodium bicarbonate (0.5 ml.) and 0.014 M sodium periodate (0.5 ml.). Samples (3 ml.) were diluted to 50 ml. for estimation but accurate results could not be obtained. It was verified that bicarbonate did not absorb at 222.5 mμ (2.5 mM bicarbonate had O.D. 0.004; cf. 0.06 mM periodate, O.D. 0.6) but interference was apparent.

Fractionation of polyhydric alcohols on Amberlite CG 400 OH^- Type I resin.

A solution (5 ml.) containing ethylene glycol, glycerol, erythritol, arabitol and mannitol (each ca. 30 mg.) was applied to a column (42 x 2.5 cm.). The eluant was supplied (125 ml./hr.) from a beaker, equipped with a magnetic stirrer and containing water (1.25 l.), to which a reservoir, consisting of an identical beaker containing ammonium carbonate solution (99 mg.; 1.25 l.) was connected by means of a syphon.

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The Position of Mannitol in Laminarin

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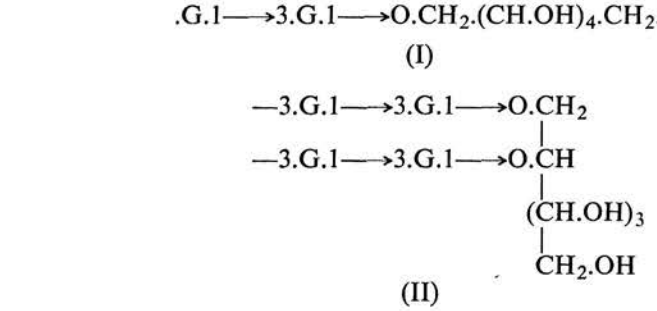
e presence of mannitol in laminarin was first ished by Peat and his co-workers[1] who isolated itol, 1-*O*-β-glucosyl-mannitol and 1-*O*-β-lamin-ɔsyl-mannitol from a partial acid hydrolysate. this and other evidence it was suggested that a ɔrtion of the laminarin molecules were non-ing and were terminated by mannitol (structure I) t others were terminated by a reducing glucose ıe. The two types of molecule have been desig-M- and G-chains respectively, and the relative ɔrtion of these in a laminarin sample can be ɛd by periodate oxidation.[2]

alternative structure (II) for the M-chains was ɔsed by Smith and his co-workers.[3] Application e reactions: periodate oxidation, reduction and ɔlysis should yield ethylene glycol from structure t not from structure (II). Since this treatment of arin apparently did not yield ethylene glycol, it concluded that the mannitol residues were stituted, as in structure (II).



(I)

(II)

an attempt to decide between these structures, arin has been oxidised at 2° with a dilute solution riodate (0·4 mM) under the conditions devised ancy and Whelan[4] for the selective oxidation of exitol portion of a disaccharide alcohol. The ıction of formic acid (calculated as mol. per itol residue) from structures (I) and (II) should and 2 mol. respectively. At 2°, formic acid is roduced from reducing 3-*O*-substituted glucose ıes.[2]

e laminarin sample examined previously[2] [degree ɔlymerisation, 24; mannitol content, 2%; pro-ɔn of M-chains, approx. 50%, and recently[5] shown to be free of mannose (*cf.* ref. 6)] gave 3·0 mol. formic acid. Two other samples of laminarin gave 3·0 and 3·1 mol. respectively, and the model substance 1-*O*-β-laminaribiosylmannitol (kindly supplied by Dr. J. R. Turvey) gave 2·9 mol. These results, which represent constant values obtained after oxidation for only one hour, are not in accord with structure (II).

We have therefore re-examined an acid hydrolysate of laminarin after periodate oxidation and boro-hydride reduction, and now report the presence of ethylene glycol. From M-chains of structure (I) and the G-chains, the laminarin hydrolysate should contain glucose (>95%), glycerol (*ca* 1–2%), ethylene glycol (*ca* 0·5%), arabitol and glycollic aldehyde. On paper chromatograms, the latter completely masked ethylene glycol. The neutralised hydrolysate from reduced polysaccharide (7·22 g.) was passed down a basic ion-exchange resin column (to remove glucose and glycollic aldehyde), and the alcohols then separated on a cellulose column[7] using butanol-ethanol-water (4:1:5, by vol.) as the solvent. Ethylene glycol (38 mg.) was finally isolated (characterised as the di-*p*-nitrobenzoate, m.p. and mixed m.p. 144–5°, and estimated from the release of formaldehyde after periodate oxidation). In view of the several manipulations involved, this yield is considered to be in good agreement with the theoretical yield (*ca* 54 mg.) for structure (I).

We conclude that in our samples of laminarin, the mannitol residues are monosubstituted as in structure (I).

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